University of Southampton

A STUDY OF THE DESMOPLASTIC REACTION IN PANCREATIC CANCER AND ITS EFFECT ON MALIGNANT PHENOTYPE

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ABSTRACT

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES
TISSUE REMODELLING AND REPAIR

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Pancreatic cancer (PC) is characterised by a profound desmoplastic reaction, which contains fibrillar collagen (collagen type I and III). This has previously been described but little is known about its origin or the functional consequence to the phenotype of PC and it was therefore investigated in the course of this thesis.

Histological examination of PCs using immunohistochemistry demonstrated gross distortion of structure of the normal pancreas. The glandular structures of the normal pancreas (surrounded by type IV collagen) were altered in PC, with wide bands of fibrotic stroma (containing type I collagen) separating the malignant glands. Numerous myofibroblastic pancreatic stellate cells (PSC) were identified throughout the desmoplastic reaction, often closely associated with cancer cells.

Potential interaction between PSC and cancer cells were tested *in vitro* using primary cultures of human PSC and pancreatic cancer cell lines (MIA PaCa-2, Panc-1 and AsPC-1). Conditioned media from each PC cell line stimulated proliferation of PSC. PSC secreted collagen in excess of the cancer cells. Furthermore conditioned media from AsPC-1 cells increased PSC procollagen I gene expression and collagen secretion (which correlated with $TGF\beta_1$ expression). PSC expressed tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) at high levels (compared to matrix metalloprotease-2) thus promoting the accumulation of extracellular matrix.

To determine the phenotypic effects of the change in collagen (type IV→type I/III) encountered in the desmoplastic reaction, pancreatic cancer cells were cultured on purified collagens (types I, III and IV). Collagen types I and III stimulated proliferation of all the cancer cells but type IV collagen and tissue culture plastic (TCP) were relatively inhibitory in comparison (except to the metastatic AsPC-1 cells). FACS analysis of the cell cycle revealed type I collagen reduced the time for cells to transit from S to G_1 phase compared to TCP. Furthermore, it was possible to block the growth stimulus to Panc-1 cells using β_1 integrin blocking antibodies.

The effect of collagen on apoptosis of pancreatic cancer cells was also studied. Type I collagen reduced the number of AsPC-1 cells undergoing apoptosis in response to 5-fluorouracil (5-FU), but had no effect in MIA PaCa-2 cells or Panc-1 cells. This was regulated by bcl-2 homologues, particularly the expression of mcl-1, which was maintained at high levels in AsPC-1 cells cultured on type I collagen. Type I collagen conferred a consistent long-term survival advantage to all 3 cancer cell lines (measured by clonogenic assay). It is likely that this was regulated by a combination of proliferative and anti-apoptotic effects mediated by type I collagen.

In conclusion, there is a detrimental interaction between pancreatic cancer cells and PSC that stimulates formation of the desmoplastic reaction abundant in type I collagen. In turn, type I collagen promotes growth of malignant cells in pancreatic cancer. This contributes to the understanding of the origin of the desmoplastic reaction by defining the interaction between pancreatic cancer cells and primary cultures of human PSC. Moreover, it demonstrates that the consequence of these interactions is deleterious to the host. This is novel in the field of pancreatic cancer and adds to the growing body of evidence implicating tumor stroma in tumourigenesis.

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Preface

The inspiration for study came from a publication by Sethi et al. in Nature Medicine in 1999, which demonstrated the importance of extracellular matrix in determining the phenotype of malignant cells. John Iredale gave me this paper in December 2000 and encouraged me to investigate the role of the desmoplastic reaction in pancreatic cancer. Pilot experiments (conducted whilst continuing to undertake surgical duties) yielded some interesting results that deserved thorough research. We were initially awarded a pump-priming grant from The Royal College of Surgeons of Edinburgh, which gave the project impetus. Hard work was subsequently rewarded by securing a comprehensive Research Training Fellowship from The Wellcome Trust. I took a leading role in developing the ideas and the program of investigation in this project and contributed as much as possible to writing the successful grant applications. I am proud of this achievement and regard it as a positive part of my research training. It is particularly gratifying that this program of research has been extended to the field of colorectal cancer, with equally interesting results. It would not have been possible to achieve this success without the appropriate guidance and supervision that I have received from the senior academics, whose support has been unwavering throughout the last 3 years.

Acknowledgements

I consider myself lucky to have been trained in research by John Iredale. He has an intuitive approach to research and always considers problems pragmatically and in the widest context of the subject matter. Furthermore, his constant optimism and zeal were always greatly appreciated.

Chris Benyon really made me think about my data and what it meant. His ability to sensibly rationalize methodological inconsistencies countered any potential nihilistic thoughts that I may have developed towards my research during the course of this study.

Graham Packham was an excellent foil to JPI and CB and often made me consider problems in a different light, not to mention providing me with considerable practical support in successfully understanding and studying apoptosis.

Colin Johnson afforded me this opportunity in the first place and has been very supportive in all my endeavours since I started working at Southampton General Hospital.

In the laboratory I must particularly thank Fanny Shek, Fiona Walker and Xiaoying Zhou for showing me the ropes when I first started. I must also thank Lindsey Murphy for undertaking the TaqMan RT-PCR experiments, Ron Lee for all the immunostaining presented in this thesis and Adrian Bateman who took the time to help me interpret it. I would also like to thank Professor Arthur who has been exceptionally supportive to my endeavors throughout my time in research.

My wife, Sarah, has provided spiritual succour throughout my research and for this I am extremely grateful.

Abbreviations

^3H	tritium
dNTPs	deoxy nucleoside triphosphates
5-FU	5-fluoruracil
7-AAD	7-amino-actinomycin D
α_3(NC1)	α_3 chain of type IV collagen
Akt	protein kinase B
Apaf	apoptosis activation factor
APC	adenomatous polyposis coli
APMA	aminophenyl mercuric acetate
aSMA	a-smooth muscle actin
ATTC	American Type Cell Culture
BCA	bionichotinic acid
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CAF	cancer associated fibroblast
cAMP	cyclic adenosine monophosphate
CBD	adenocarcinoma of the common bile duct
CCK	cholecystokinin
CD	cluster of differentiation
CDK	cyclin dependant kinase
CDKI	cyclin dependant kinase inhibitor
cDNA	complimentary deoxyribose nucleic acid
CTGF	connective tissue growth factor
DC	duodenal cancer
dH_2O	deionised water
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribose nucleic acid
DR	death receptor
dsDNA	double stranded DNA
DTT	dithiothreitol
ECACC	Eurpean Collection of Cell Cultures
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunosorbent assay
EMMPRIN	extracellular matrix metalloproteinase inducer
ERK	extracellular signal related kinase
FACS	fluorescence activated cell sorter
FAK	focal adhesion kinase
FAP	familial adenomatous polyposis
FCS	foetal calf serum
FGFa	fibroblast growth factor (acidic)
FGFb	fibroblast growth factor (basic)

FITC	flourescein isothiocyanate
FLICA	flourochrome inbibito of caspases
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDP	guanidine diphosphate
GFAP	glial fibrillary acid protein
GIT	guanidine isothiocyanate
GTP	guandine triphosphate
HBSS -	Hank's balanced salt solution (without calcium)
HBSS +	Hank's balanced salt solution (with calcium)
HCl	hydrochloric acid
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanosulfonic acid)
HRP	horseradish peroxidase
HSC	hepatic stellate cell
HSPG	heperan sulphate proteoglycan
HSP	heat shock protein
ICAP	integrin cytoplasmic-domain associated protein
Ig	immunoglobulin
IGF	insulin like growth factor
Il	interleukine
IPMT	intraductal papillary mucinous tumour
KGF	keratinocyte growth factor
MAPK	mitogen associated protein kinase
MEK	mitogen activated protein kinase
MMP	matrix metalloproteinase
MMPI	matrix metalloproteinase inhibitor
Mn2+	manganese ions
MOPS	morpholino propanosulfonic acid
mRNA	messenger ribose nucleic acid
NaOH	sodium hydroxide
NEM	N-ethylmaleimide
NGF	nerve growth factor
PAGE	polyacrylamide gel electrophoresis
PanIN	pancreatic intraepithelial neoplasia
PARP	poly (ADP-ribose) polymerase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PC	pancreatic cancer
PDAC	pancreatic ductal adenocarcinoma
PDGF	platelet derived growth factor
PI	propidium iodide
PI3K	phosphinositide 3-kinase
PSC	pancreatic stellate cell
PSG	penecillin/streptomycin/gentamycin
Raf	Raf kinase
Rb protein	retinoblastoma protein
RER	rough endoplasmic reticulum
RGD	arginine-glycine-aspartic acid (amino acid sequence)
RNA	ribose nucleic acid
RNAse	ribonuclease

SDS	sodium dodecyl sulphate
TACP	tumour associated chronic pancreatitis
TCA	trichloroacetic acid
TCP	tissue culture plastic
TE	Tris/EDTA
TEMED	N'N'N'N'-tetramethyletheylenediamine
TGF	transforming growth factor
TIMP	tissue inhibitor of matrix metalloproteinase
TMB	tetramethyl benzidine
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
Tris	Tris[hydroxymethyl]aminomethane
TS	tris saline
TST	0.05%Tween 20v/vTS
VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal peptide

Chapter 1
Literature Review

Part 1
Biology of Epithelial Cancers

1. General

Cancer cells are exceptionally rare. A human body contains 10^{14} cells, many of which undergo frequent mitosis, yet a cancer arises in only 1 in 3 lifetimes (Evan and Vousden, 2001). It is the combination of deregulated cell proliferation and resistance to apoptosis that are the hallmark of neoplasia (Evan and Vousden, 2001). The observation that certain malignancies are associated with exposure to particular agents has led to the discovery of mechanisms underlying abnormal cell growth in malignancy (Stevens and Lowe, 1995a). Carcinogenic agents are diverse: chemicals, viruses, irradiation, physical agents or other processes such as chronic inflammatory disease can all lead to neoplasia (Stevens and Lowe, 1995a). The end point of the process is essentially the same: deregulated cell growth caused by abnormalities in genes that control cell proliferation and cell death (Stevens and Lowe, 1995a; Hanahan and Weinberg, 2000). Abnormalities in these genes can be caused by point mutations (within the gene), gene amplification or chromosomal rearrangements resulting in inappropriate gene activation by another promotor region (Stevens and Lowe, 1995a). The normal mechanisms controlling cellular proliferation and apoptosis are discussed below. The manner in which DNA damage permits malignant growth is also outlined. Carcinogenesis in pancreatic cancer is discussed in Parts 3 and 5.

2. Cell Proliferation

Cells undergo mitosis in response to specific signals. The cell cycle is divided into four sequential phases: G_1, S phase, G_2 and M phases (Fig. 1.1). A functioning cell not in active cycle is described as being G_0 (Alberts et al., 1994). As with most biological processes mitosis will occur if the balance of pro-mitotic signals outweighs the anti-mitotic signals. Mitosis is driven by cyclin dependent kinases (CDKs)(Grana and Reddy, 1995). Their activity is regulated by the availability of cyclins and the presence of cyclin dependent kinase inhibitors (CDKIs) (Grana and Reddy, 1995). CDKs act in G_1 by phosphorylating retinoblastoma protein (Rb) and by so doing, release E2F transcription factors (Grana and Reddy, 1995). Once

released from Rb, E2F transcription factors stimulate the transcription of genes required to enter S phase (Grana and Reddy, 1995). Checkpoints exist within the cell cycle, predominantly triggered by DNA damage/errors or evidence of ongoing DNA repair, which prevent genetically abnormal cells replicating (Nurse, 2000).

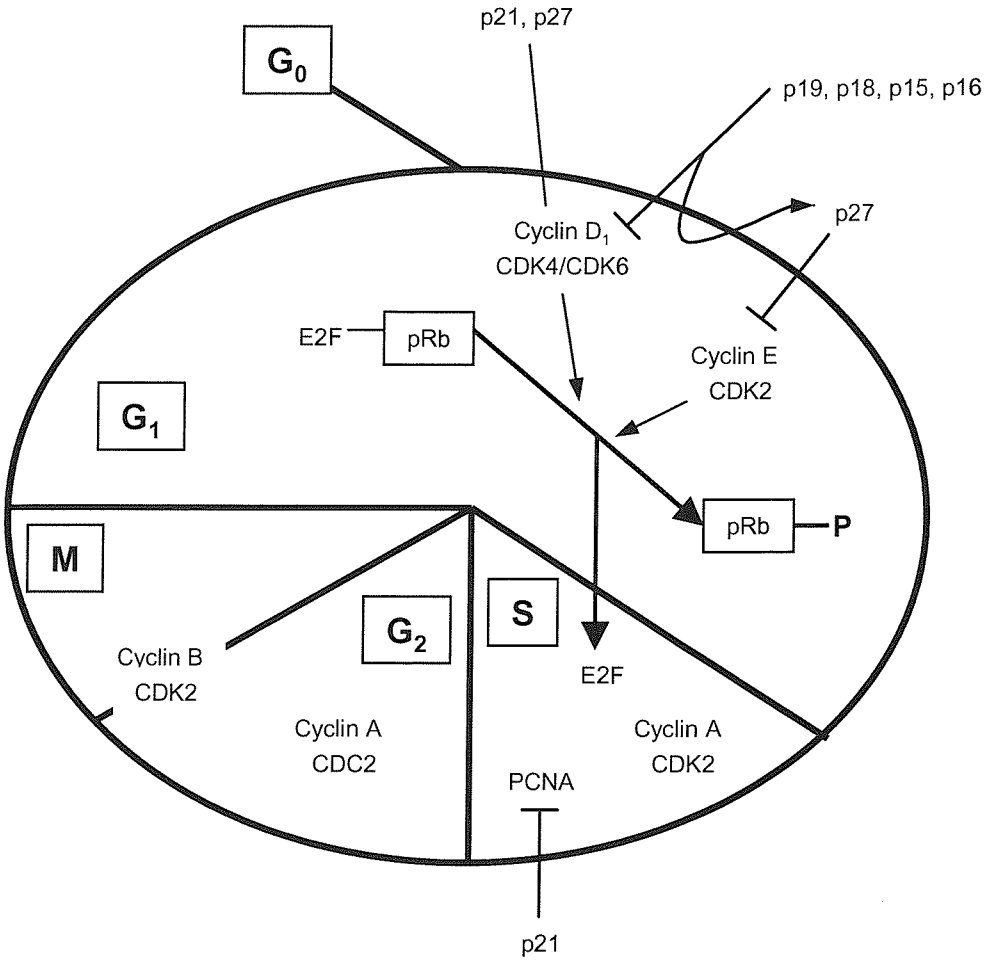


Figure 1.1
Molecular regulation of the cell cycle (Poon, 2002)
CDK (cyclin dependant kinase) and PCNA (proliferating nuclear antigen)

Perhaps most important in malignancy is the G_1 checkpoint that prevents cells entering S phase, known as the Restriction Point (Carnero, 2002). In simple terms, the cell cycle becomes deregulated by abnormal activation of the pro-mitotic 'machinery' or the dysfunction of CDKIs, which allows a cell to pass through the restriction point (Carnero, 2002). These characteristics are the essence of a malignant cell. Genes that promote cell division (by the mechanisms described above) are known as proto-oncogenes and genes that suppress cell division/promote cell death are termed tumour suppressor genes. It is functional errors or failure of appropriate expression of these genes, which result in the uncontrolled cellular proliferation that characterises malignancy, although as

demonstrated later, dysfunction of apoptosis also contributes (Evan and Vousden, 2001).

3. Mechanisms of Deregulated Cell Proliferation

Oncogenes are derived from proto-oncogenes. In healthy cells the gene products of proto-oncogenes fulfil complex roles and may lead to both cell division and apoptosis (Evan and Vousden, 2001; Sears and Nevins, 2002; Steiner et al., 1996). Because of this bi-directional effect, mutations to proto-oncogenes often require mutations to tumour suppressor genes (e.g. p53), making the cell resistant to apoptosis for autonomous, malignant growth to occur. Oncogenic mutations permit cellular growth independent of external growth signals and regardless of external inhibitory signals (Hanahan and Weinberg, 2000). Such interactions need to be accompanied by limitless replicative potential, conferred to all types of malignant cell through telomere maintenance (Hanahan and Weinberg, 2000). This concept underlies the 'two-hit' hypothesis of carcinogenesis defined by Knudson and applies to the development of pancreatic cancer (Knudson, 2001; Bardeesy and DePinho, 2002).

Important Oncogenes

- Myc: Myc proteins are transcription factors that can activate and repress target genes. Their expression is tightly regulated in the cell and this is partly facilitated by their short half-life. Mutations/deregulation of Myc genes can reduce growth factor dependence of cells by activation of CDKs and activation of E2F dependent transcription (bypassing Rb phosphorylation). Cells may also become resistant to anti-mitogenic factors under the control of Myc. For example, transforming growth factor β (TGFβ) induces CDKIs but the function of these may already be suppressed by deregulated Myc gene expression. Myc genes have been found to promote cellular immortalisation, genomic instability (by destabilising checkpoint mechanisms) and angiogenesis, although the exact mechanisms by which these occur remain elusive (Lutz et al., 2002; Evan and Vousden, 2001).
- Ras: Ras protein heads a series of signalling pathways that transduce growth signals from cell surface receptors. The functional properties of Ras are complex. Oncogenic activation of Ras occurs with mutations that maintain Ras in guanosine triphosphate (GTP) bound (activated) form. Such mutations occur in 70% of cancers and render cell survival much less dependent on external stimuli. The three main intracellular signalling pathways downstream to Ras are:

RAF/MEK/ERK, Ral and PI3K/Akt (see figure 1.2) (Macaluso et al., 2002; Sears and Nevins, 2002).

- EGFR/c-erbBs: The EGF family of receptors and ligands may act as oncogenes if they are over expressed. Growth factor/receptor over-expression is oncogenic because it produces an abnormal persistent growth stimulus to cells, without feedback regulation (Ghaneh et al., 2002).

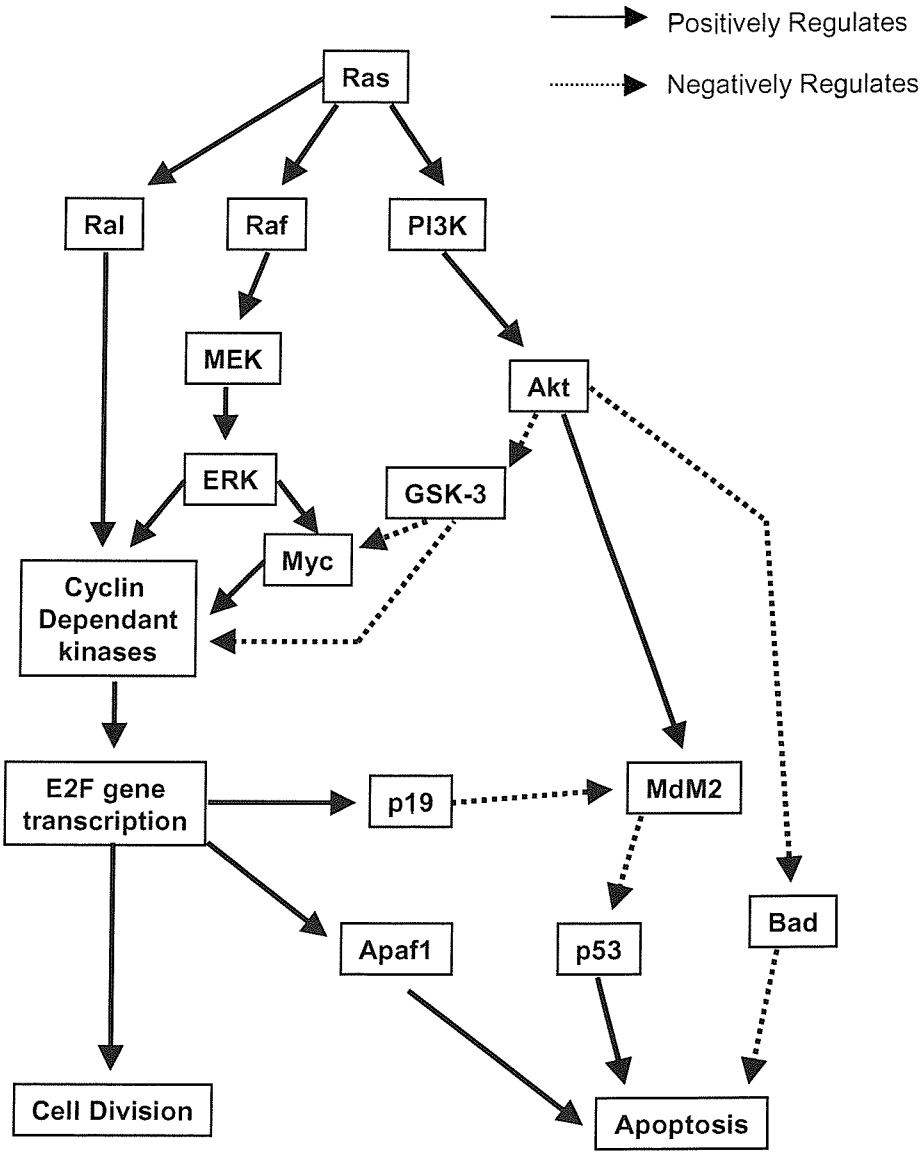


Figure 1.2
Multiple Ras effector pathways and the integrated role of Myc (Sears and Nevins, 2002)

Important Tumour Suppressor Genes

Mutations to genes that regulate either the propagation of cell cycle or initiate apoptosis in response to harmful stimuli have the potential to be pro-malignant, the classic example being p53 (Ho and Dowdy, 2002). CDKIs have important tumour suppressor duties, of which there is the INK4 family (p16, p15, p18, p19) that inhibit specific CDKs and the KIP family (p21, p27, p57) which are less specific and act in a concentration dependent manner (Carnero, 2002). Other factors that are inhibitory to growth also have tumour suppressor functions (e.g. TGFβ).

- p53: p53 is known as the' guardian of the genome', because it prevents proliferation of damaged cells. It is induced by DNA damage, hypoxia, oxidative stress, and excessive mitogenic stimuli amongst others, all of which are encountered in malignancy. Its importance is underlined by the high frequency of p53 mutations found in malignant tumours. When mutated, damaged cells continue to proliferate rather than undergoing p53 induced apoptosis (Sharpless and DePinho, 2002).
- p16: This protein normally inhibits CDKs 4 and 6 associating with cyclin D and by doing so prevents subsequent transcription of E2F genes that leads to S phase entry. Mutation of p16 disables the G_1 checkpoint (Ghaneh et al., 2002).
- p21: p21 is a downstream effector of p53 that prevents phosphorylation of Rb and subsequent cell cycle progression. It is therefore essential for G_1 cell cycle arrest (Xiong et al., 1993).
- TGFβ: The normally inhibitory effects of TGFβ in cell proliferation are mediated via TGFβ receptors (of which there are 3) and intracellular transduction via the Smad pathway. Dysfunction at any point in the pathway releases cells from the inhibitory effects of TGFβ (Korc, 1998).

4. Apoptosis

Apoptosis is a physiological form of cell death that can be considered to have 3 phases: a death signal, cellular execution and phagocytosis of debris. Apoptosis can also be regarded as a physiological default, whereby cells remove themselves from tissues in the absence of appropriate survival signals (Hengartner, 2000). Cellular fate is determined either by intracellular (e.g. in the event of DNA damage) or intercellular death signals (e.g. from lymphocytes). When a death signal occurs, a series of coordinated events take place leading to destruction of the cell, summarised in figure 1.3. (Hengartner, 2000). Morphologically this is characterised by nucleosomal condensation and fragmentation and membrane blebbing/budding.

This process culminates in the formation of apoptotic bodies (Stevens and Lowe, 1995b). This process takes 20-30 minutes and ends with phagocytosis of apoptotic bodies by macrophages and other specialised phagocytes (Stevens and Lowe, 1995b).

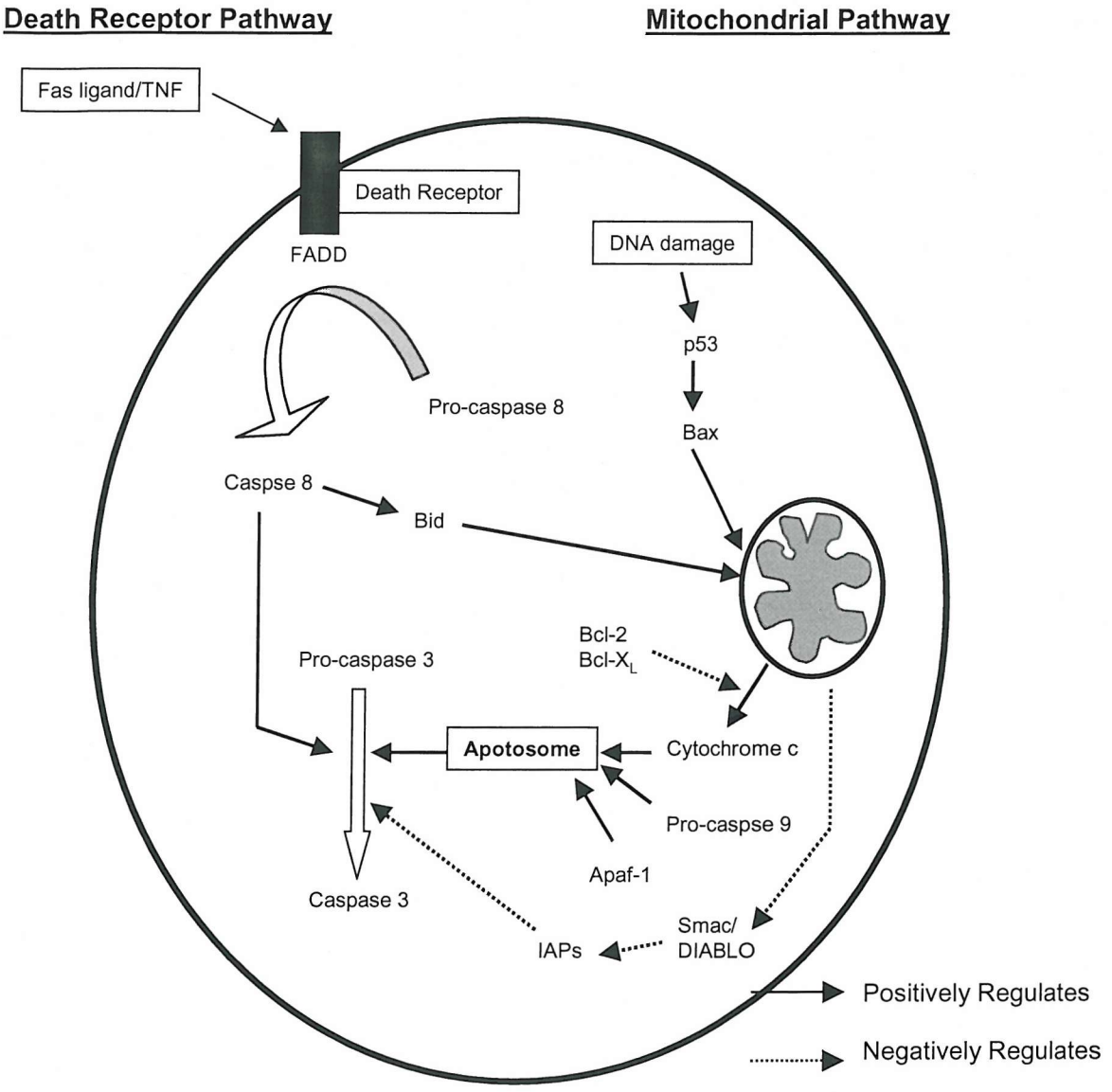


Figure 1.3
Pathways controlling apoptosis (Hengartner, 2000)

Enzymes called caspases are central to cellular execution and are highly conserved throughout different species. They selectively cleave target proteins that in turn degrade substrates, such as DNA, in a characteristic fashion. Caspases can be viewed as either initiator caspases or effector caspases. Caspases 8 and 9 initiate apoptosis. Caspase 8 is thought to become activated by forced crowding (a sort of critical mass phenomenon as caspase 8 accumulates at the receptor), in response to specific signalling via death receptors. Pro-caspase 9 on the other hand requires

binding of a dedicated, cytoplasmic co-factor protein called Apaf-1 and also cytochrome c released from mitochondria. This complex is known as an apoptosome. Effector caspases (3, 6 and 7) are then proteolytically cleaved by initiators, leading to an integrated and amplified cellular death signal.

As alluded to above, there are two pathways by which apoptosis is activated. The death receptor pathway is classically initiated by the Fas receptor (CD95/Apo-1) but there are a number of other receptors (Trail-R1, Trail-R2, DR6, DR3, TNF-R1) each of which have multiple names assigned to them. Their ligands include Trail, TNFα and Fas amongst others. Receptors and ligands each exhibit remarkable homology. The mitochondrial pathway is initiated by the release of cytochrome c from mitochondria. This process is tightly controlled by the bcl-2 family of proteins. The family is split into pro-apoptotic (including Bax, Bak and Bid) and anti-apoptotic members (including Bcl-2, Bcl-X_L and mcl-1). It is the balance in expression of these proteins that controls the release of cytochrome c. Malignant cells escape normal regulation by Bcl-2 homologues by over expression of anti-apoptotic homologues or a failure in regulation of pro-apoptotic homologues (e.g. by mutated p53 in response to DNA damage) (Hengartner, 2000).

5. Mechanisms of Deregulated Cell Survival

In health, most cells are dependent on anchorage to extracellular matrix and other cells as well as soluble trophic factors. Different cells have different requirements but such requirements confine cells to a distinct location(Evan and Vousden, 2001). Epithelial cells are dependant on anchorage to extracellular matrix (basement membrane) and if they become detached they undergo apoptosis, specifically termed anoikis (Howe et al., 2002). Deregulation of this process is essential to metastasis and therefore suppression of apoptosis or the development of anchorage independent growth is essential to the malignant phenotype (Hanahan and Weinberg, 2000). There are a number of ways by which this occurs. One is over expression of survival signals such as insulin-like growth factors (IGFs), leading to autocrine stimulation. Another is mutation in cytoplasmic signalling proteins such as Akt or Ras. Integrins signal via the Ras pathway and abnormal activation of this pathway in Ras mutations may provide sufficient survival signals for the cell to avoid apoptosis on detachment from extracellular matrix (Sears and Nevins, 2002). Anti-apoptotic proteins such as Bcl-2 and Bcl-X_L are inappropriately over expressed in a number of cancers preventing cytochrome c release from mitochondria. Apaf-1 has been found deleted in other tumours (Evan and Vousden,

2001). Perhaps the most important of all is mutation to p53 (Sharpless and DePinho, 2002). Hence there are multiple mechanisms by which tumour cells become resistant to apoptosis (Evan and Vousden, 2001; Hanahan and Weinberg, 2000).

6. Other prerequisites for successful oncogenesis

In order for malignant cells to form tumours, neoplasms must be able to elicit sustained angiogenesis and possess the ability to invade tissues and metastasise (Hanahan and Weinberg, 2000).

Part 2

The Pancreas

1. Macroscopic structure

The pancreas is a retroperitoneal organ, situated immediately posterior to the lesser sac. It is approximately 15cm long and lies across the vertebral column. The pancreatic head overlies the vena cava and aorta to the right and the tail of the pancreas extends to the hilum of the spleen on the left. The head of the pancreas is cradled by the duodenum, to which it is intimately related (McMinn, 1995).

The pancreas has a rich blood supply, predominantly derived from the splenic artery and pancreaticoduodenal arteries. Venous blood drains directly into the superior mesenteric and splenic veins, which form the portal vein immediately posterior to the pancreatic head. Lymphatic drainage is directed into retropancreatic, superior mesenteric and coeliac nodes. The pancreas is innervated by parasympathetic vagal fibres and sympathetic nerves derived from thoracic cord segments 6-10 (McMinn, 1995).

The rich blood supply and close anatomical relationship with the foregut reflect the multiple and integrated roles that the pancreas plays in digestion and metabolism but presumably contribute to the early local invasion and metastasis, characteristic of pancreatic cancer.

2. Microscopic structure and function

The pancreas is a lobulated organ, with each lobule containing numerous acini. The gland is surrounded by a delicate capsule of connective tissue, from which fine septa enter the gland, providing support for the parenchymal elements. The septa

act as conduits for larger blood vessels and nerves, as well as housing the stromal cells of the pancreas (McMinn, 1995).

Exocrine pancreas

The functional cells of the pancreas are divided into exocrine (acinar and ductal cells) and endocrine (Islets of Langerhans) types. Cells of the pancreatic acinus synthesise digestive enzymes and this is reflected in abundant rough endoplasmic reticuli (RER) and zymogen granules within the cytoplasm. Cell morphology gradually changes towards the small intercalated pancreatic ducts, where centroacinar cells provide the transition to ductal epithelia. Centroacinar cells are less basophilic than acinar cells because they contain less RER. The ductal epithelium is responsible for bicarbonate rich secretions and adenocarcinomas of the pancreas often have a ductal phenotype (Ross et al., 1989a; Real, 1996). Secretion of enzymes and bicarbonate is under the control of the vagus nerve and two hormones secreted by the duodenum. Secretin is released as fats or gastric chyme enters the duodenum. This results in the secretion of bicarbonate by ductal cells until pH is neutralised allowing the enzymes to function. Cholecystokinin (CCK) is also released when food enters the duodenum and this results in secretion of enzymes (Despopoulos and Silbernagl, 1991).

Endocrine Pancreas

The Islets of Langerhans are scattered throughout the pancreas but are more numerous in the tail. The cells secrete various hormones including insulin (B cells), glucagons (A cells) and gastrin (G cells) and are arranged in irregular cords (Ross et al., 1989a). These cells may themselves undergo neoplastic transformation and from endocrine tumours that can lead to an interesting variety of clinical syndromes related to excessive uncontrolled hormone secretion (Russell, 1998). More commonly B cell failure causes diabetes.

Part 3
Clinical Aspects of Pancreatic cancer

Pancreatic ductal adenocarcinoma (PDAC) is almost without exception fatal and there has been little impact made on this in the last 25 years.

1. Incidence

Pancreatic cancer is the 5th commonest cancer and accounts for approximately 5500 deaths/year in the England (Cancer Statistics Registration, 1998).

2. Aetiology

Pancreatic cancer is a disease of advancing age, with the risk for an individual increasing 40 fold between the ages of 40 and 80 (Bardeesy and DePinho, 2002). This implicates environmental factors but the only significant environmental risk factor is smoking, with a relative risk of 2 (Russell, 1998). Other factors such as alcohol, can indirectly increase the risk of pancreatic cancer, as a causative factor of chronic pancreatitis. Sporadic chronic pancreatitis is a disease characterised by chronic inflammation that leads to pancreatic fibrosis, with gradual loss of functional pancreatic tissue. It is associated with 17-26.7 fold increased risk of developing pancreatic cancer (Lowenfels and Maisonneuve, 2002; Stevens and Lowe, 1995a; Malka et al., 2002). There is also a heritable form of chronic pancreatitis, in which there is a 50 fold increased risk of developing pancreatic cancer with an affected individual having a 30% lifetime risk.

Mechanisms underlying the role of inflammation in the development of pancreatic cancer have recently been comprehensively reviewed, yet the change in quality of matrix in chronic pancreatitis was not discussed because of a paucity of information (Farrow and Evers, 2002).

3. Diagnosis

Patients classically present with a combination of vague abdominal pain, weight loss and jaundice, as the common bile duct is compressed during its passage through the head of the pancreas. If clinically suspected, the diagnosis is usually made with ultrasound or computed tomography (CT scanning).

4. Treatment

At presentation only 10-15% of patients are suitable for surgery (Warshaw et al., 1990). The rest have locally advanced or metastatic disease or are simply not fit enough to undergo major surgery. The standard operation performed is a pancreatico-duodenectomy (Whipples procedure), which involves removal of the head of the pancreas, lower bile duct and duodenum. This is rarely curative but offers the best palliation from the pain and duodenal obstruction of advanced pancreatic cancer. Adjuvant treatment with 5-fluorouracil has recently been shown to improve survival after surgery, with a 5 year survival of 23% (Neoptolemos et al., 2003). Jaundice is palliated by endoscopic retrograde stent placement (Russell, 1998).

Chemotherapy is also given on a palliative basis. There are a number of regimens based on 5-fluorouracil (or newer derivatives) but none has had a response rate of more than 20% either objectively in terms of tumour shrinkage or subjectively in terms of symptomatic relief (Ahlgren, 1996).

5. Prognosis

The approximate median survival time is 13-17 months in those who undergo pancreatic resection (increased to 21.6 months with adjuvant chemotherapy) and 6 months in those who receive the best medical management (Schafer et al., 2002; Neoptolemos et al., 2003). Overall the best summary lies in the statistics: death:incidence ratio=0.99 (Devesa et al., 1995).

These statistics underline the need for novel treatment strategies in the management of pancreatic cancer.

Part 4

Regulation of Extracellular Matrix in Health and Disease

One common feature of pancreatic ductal adenocarcinoma is a desmoplastic reaction in which there is development of extensive fibrosis in the tumour mass, akin to scar tissue. The importance of the human wound healing response in cancer was emphasized by a much-cited paper, written by Dvorak in 1986 (Dvorak, 1986). In it he likens cancer to wounds that do not heal in which cancer cells subvert the wound healing response to further their own growth and form tumours.

1. Normal stroma

Stroma is a complex of stromal cells, extracellular matrix, blood vessels, nerves and lymphatics. In organs such as the pancreas stroma provides the structural framework on which the metabolically functional elements rest. Epithelial cells are anchored to basement membrane, which is a meshwork principally comprised of type IV collagen and laminin (see figure 1.3). Beneath the basement membrane lies the interstitial stroma, the main structural elements of which are fibrillar collagens (types I and III predominantly) that provide tensile strength to tissues. Large, hydrophilic proteoglycans are also found in the interstitium. These trap water and confer turgor to the interstitial matrix.

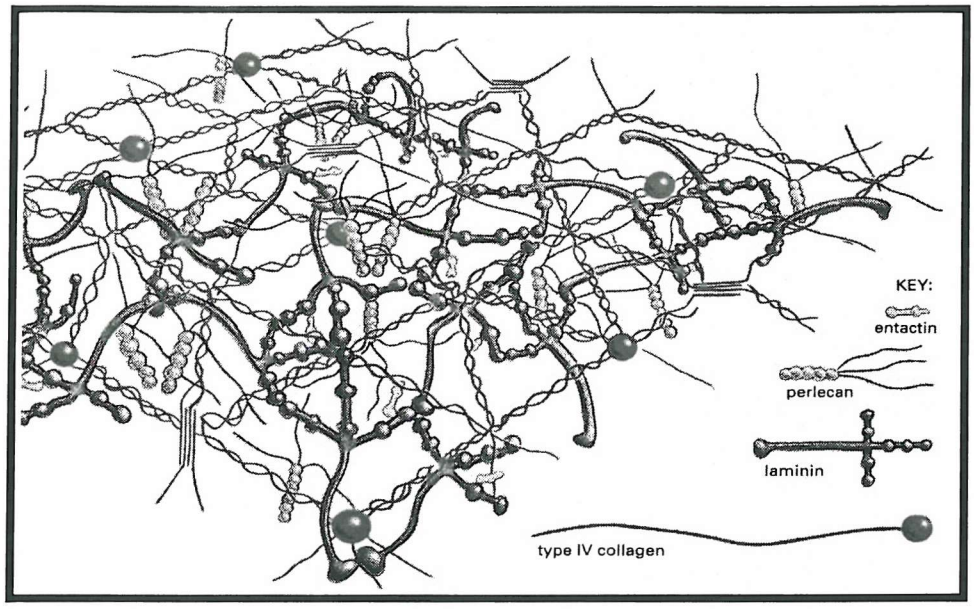


Figure 1.4
Basic structure of basement membrane
Basement membrane is principally comprised of collagen type IV and laminin (reproduced from (Alberts et al., 1994))

Throughout the extracellular matrix growth factors and cytokines are contained, only being released by processes that remodel tissues. Interstitial extracellular matrix is synthesised by stromal cells such as fibroblasts and myofibroblasts and the interstitial stroma is normally punctuated by such cells. Other cell types that occupy the interstitium include immune cells such as macrophages, although the exact complement of immune cells depends on tissue type (Ross et al., 1989b; Ross et al., 1989c; Alberts et al., 1994).

2. Stromal response to injury

The stromal reaction to injury and subsequent wound healing is complex but relevant to tumours (Dvorak, 1986). Following wounding of a tissue, a blood clot forms, providing basic matrix (fibrin) for cellular and vascular invasion. The area is gradually infiltrated by fibroblasts, myofibroblasts (which secrete collagen, fibronectin and proteoglycan) and macrophages (which initiate phagocytosis of dead tissue and secrete growth factors) and a fragile network of new capillaries develops, replacing the area of damaged tissue. This is known as *vascular granulation tissue.* As this matures the balance changes, favouring deposition of collagen by myofibroblasts, loss of macrophages and regression of capillaries to form *fibrovascular granulation tissue.* This process continues until there are relatively few vascular channels, with collagen deposition in line with tensile forces. This is called *fibrous granulation tissue* and with time the myofibroblastic cell

content reduces and mature scar tissue is seen (Mutsaers et al., 1997; Stevens and Lowe, 1995c; Cotran et al., 1989a).

3. Matrix turnover

There is a balance between extracellular matrix being synthesised by cells such as myofibroblasts and being degraded by proteolytic enzymes. The major enzyme family that regulate extracellular matrix degradation are called matrix metalloproteinases (MMPs) (Nagase and Woessner, Jr., 1999). Their main function in health is removal of extracellular matrix during tissue reabsorption (wound healing, bone remodelling and uterine/placental interaction during pregnancy) (Nagase and Woessner, Jr., 1999). Of equal importance is the many key roles they play in diseases ranging from arthritis to cancer (Nagase and Woessner, Jr., 1999).

MMPs are calcium dependent enzymes that are secreted in an inactive proenzyme form (Nagase and Woessner, Jr., 1999). There is an N-terminal pro-domain, a catalytic domain and a C-terminal hemopexin like domain. The pro-domain inactivates the catalytic domain, until cleaved off in the process of activation. The cleavage site is highly conserved among MMPs (Nagase and Woessner, Jr., 1999). There are 25 known members of the MMP family and table 1.1 shows the various functional groups of the family and their substrates (Benyon and Arthur, 2001).

MMP are synthesised by stromal and cancer cells in malignancy and their activity is governed by gene transcription/translation, pro-enzyme activation and extracellular inhibition (Egeblad and Werb, 2002). A diverse range of stimuli control MMP activity but inflammation is central in regulating matrix turnover (Benyon and Arthur, 2001).

Inflammatory mediators are implicated in the induction of MMP gene expression: TNFα, Il-1 and FGF increase synthesis of certain MMPs (e.g. MMP-1 and MMP-3) (Benyon and Arthur, 2001; Nagase and Woessner, Jr., 1999). Other factors, such as TGFβ, glucocorticoids and retinoic acid derivatives generally down regulate MMP gene (Benyon and Arthur, 2001; von Marschall et al., 1998). Novel mechanisms of MMP induction are regularly being discovered, particularly in the context of disease. One such example is a cell membrane bound glycoprotein called extracellular matrix metalloproteinase inducer (EMMPRIN) (Zucker et al., 2001). This can be found in healthy tissues but is expressed at very high levels in

certain cancers, which may partly explain the apparent excess of MMPs in certain tumours (Zucker et al., 2001).

Inflammation is also important in activation of pro-MMPs. Pro-MMP-3 is directly activated by factors secreted by mast cells and neutrophils. Plasmin is also able to activate pro-MMPs 2 and 3 (Benyon and Arthur, 2001). Integrin dependent anchorage to specific extracellular matrices can influence MMP activity; type I collagen indirectly activates MMP-2 by activating MT-1 MMP (which then activates MMP-2 see below), whereas type IV collagen inhibits activation of MT1-MMP (Ellerbroek et al., 2001; Ivaska and Heino, 2000; Pasco et al., 2000a). Once activated, certain MMPs activate others (e.g. MMP-3 activates MMP-1) (Benyon and Arthur, 2001). MT1-MMP is membrane bound and thought to be activated via intracellular signals. It not only has intrinsic collagenolytic activity, but also binds and activates soluble MMP-2 (in a complex associated with tissue inhibitor of matrix metalloproteinases-2 (TIMP-2)). This is important because MMP-2 is known to degrade basement membrane and has been implicated in the malignant phenotype of many tumours (Egeblad and Werb, 2002).

Activated MMPs can be subject to inhibition. TIMPs are specific inhibitors that neutralise MMPs in an equimolar ratio. TIMP-1 neutralises all MMPs but the membrane bound types (e.g. MT-1 MMP) less efficiently. It is induced by the profibrogenic growth factor TGFβ. TIMP-2 plays a specific role in MMP-2 activity, as mentioned above (Gomez et al., 1997). The roles of TIMP-3 and 4 are less well defined. MMPs are also inhibited by α2-macroglobulin, a non-specific serum proteinase inhibitor (Benyon and Arthur, 2001).

Simplistically it is the balance between these various factors that determines if matrix accumulates or disappears. It is very important, however, to appreciate that MMPs have been found to have numerous other roles in tissue haemostasis. They regulate cell growth, apoptosis, angiogenesis, cellular invasion (and metastasis in cancer) and immune responses, all of which are central to tumour behaviour, including pancreatic cancer (Egeblad and Werb, 2002; Bramhall, 1997). TIMPs are also complex in their functions. TIMP-1 for example acts as a survival factor for a number of cell types through subtle changes to proliferation and apoptosis (Hayakawa et al., 1992; Murphy et al., 2002; Guedez et al., 1998). Current dogma suggests that TIMPs benefit the host by inhibiting MMPs in cancer, but this is likely

to be an over simplistic view and will be discussed in the context of pancreatic cancer later (Egeblad and Werb, 2002).

The stromal reaction to injury is a complex one but it is very important in the context of malignancy because this response may inadvertently enhance tumour formation.

Classification		Known matrix substrates
Interstitial Collagenases		
MMP-1	interstitial collagenase	collagen types III, I, II, VII, VIII, X
MMP-8	neutrophil collagenase	collagen types I, III, II
MMP-13	collagenase-3	collagen types II, III, I, VII, X
Gelatinases		
MMP-2	gelatinase A	collagen V, IV, I, VII, X, gelatins, elastin, laminin
MMP-9	gelatinase B	
Stromolysins		
MMP-3	stromolysin-1	collagen types III, IV, V, IX, gelatins, fibronectin, proteoglycans, laminin, (activates MMP-1)
MMP-7	matrilysin	entactin, gelatin, elastin, fibronectin, vitronectin, laminin, fibrinogen
MMP-10	stromolysin-2	as MMP-3, elastins
MMP-11	stromolysin-3	weak matrix degrading activity
Membrane-type		
MMP-14	MT1-MMP	collagen types I, II, III, fibronectin, vitronectin, fibrinogen, (activates MMP-2 and MMP-13)
MMP-15	MT2-MMP	fibronectin, tenascin, laminin, aggrecan, perlecan, (activates MMP-2)
MMP-16	MT3-MMP	collagen III, fibronectin
MMP-17	MT4-MMP	fibrinogen, fibrin
MMP-24	MT5-MMP	proteoglycans (activates MMP-2)
MMP-25	MT6-MMP	collagen type IV, gelatin, fibronectin, fibrin
Metalloelastase		
MMP-12	macrophage elastase	elastin, gelatins, collagen type IV, fibronectin, proteoglycan

Table 1.1
Classification of MMPs and their substrates (Benyon and Arthur, 2001)

Part 5

Basic Pathology of Pancreatic Cancer

1. Cellular origin of Pancreatic Adenocarcinomas

Until recently there has been no well-defined adenoma-carcinoma sequence or metaplasia-dysplasia-carcinoma sequence characterised in the pancreas. Nearly all pancreatic adenocarcinomas display a phenotype suggestive of a ductal origin (Real, 1996; Cruickshank, 1986). Evidence from animal models suggests acinar cells may transdifferentiate, adopting ductal phenotype in the process of carcinogenesis, which prevents definitive identification of cellular origin (Wagner et al., 1998a). However other authors present a strong case for ductal adenocarcinomas originating from islet cells (which have been shown to have remarkable potential for transdifferentiation) (Pour et al., 2003). Hyperplastic and regenerative lesions are observed in the ductal epithelium of resected human specimens, removed for cancer and chronic pancreatitis (Williams et al., 1996). These are thought to represent pre-malignant lesions and have been systematically classified in the Pancreatic Intraepithelial Neoplasia (PanIN) classification, through which the study of malignant ductal cell transformation should be furthered (Luttges and Kloppel, 2000; Klein et al., 2002; Hruban et al., 2001). Intraductal-papillary mucinous tumours (IPMTs) are a distinct entity from PanIN lesions (based on molecular analysis) and are also recognised to be pre-malignant lesions in the pancreas (Luttges and Kloppel, 2000). In common with other organs, chronic inflammatory conditions within the pancreas dramatically increase the incidence of malignant change(Lowenfels and Maisonneuve, 2002; Stevens and Lowe, 1995a).

2. Genetic and molecular abnormalities in pancreatic cancer

Hereditary chronic pancreatitis results in a 30% lifetime risk of developing pancreatic cancer (Lowenfels and Maisonneuve, 2002). The underlying genetic abnormalities are mutations to the trypsinogen gene on chromosome 7q35 and BRCA-2 genes (Whitcomb, 2000; Bardeesy and DePinho, 2002). This is thought to lead to inappropriate or enhanced trypsinogen activation, causing acinar cell autolysis, with the inevitable fibrosis that ensues (Farrow and Evers, 2002). Pancreatic cancers often demonstrate extensive genomic instability, aneuploidy and telomere attrition (Bardeesy and DePinho, 2002). Studies of resected pancreatic cancers show many of the common mutations to proto-oncogenes and tumour suppressor genes that are found in other tumours. These include activating K-*ras* mutations (75-90% of tumours), p53 alterations (65%), p16 inactivation (up to

85%), p21 mutations and cyclin D1 over expression (65%) (Ghaneh et al., 2002). p53 mutations alone, correlate to a worse prognosis but none of the others have been shown to be prognostic indicators (Ghaneh et al., 2002). Mutations to retinoblastoma protein, myc or APC genes are rarely detected in pancreatic cancer although cMyc has recently been reported as over-expressed in up to 43% of tumours (Schutte and Kern, 1996; Schleger et al., 2002). There appears to be a progressive accumulation of abnormalities as the phenotype evolves from benign to malignant (Bardeesy and DePinho, 2002; Pour et al., 2003; Knudson, 2001).

Pancreatic cancer cells are resistant to death receptor mediated apoptosis, despite being well furnished with receptors (Elnemr et al., 2001; Hinz et al., 2000; Ozawa et al., 2001). A number of molecular abnormalities have been cited as responsible for this, including over-expression of Bcl-X_L, Fas decoy receptors and FLICE inhibitory protein (Hinz et al., 2000; Elnemr et al., 2001). The mitochondrial apoptotic pathway may not function normally either. Studies have found variable expression of Bcl-2 family proteins but only over expression of Bcl-X_L correlates with a worse prognosis (Evans et al., 2001).

3. Histological characteristics of pancreatic adenocarcinoma

When an adenocarcinoma develops in the pancreas it leads to a focal mass. This may result in chronic blockage of pancreatic ducts and a degree of tumour associated chronic pancreatitis (TACP) may develop around the tumour and in the pancreas distal to the tumour (Imamura et al., 1995a). At a macroscopic level, the mass may well be visible and as a result of the TACP the pancreas may be slightly tense and inflamed.

At a microscopic level there are dramatic changes to pancreatic structure. The degree to which this occurs depends on the degree of tumour differentiation and stromal reaction. Pancreatic cancer is characterised by a very marked, *fibrous* desmoplastic reaction (see Chapter 3) (Cruickshank, 1986). Tumours are described as desmoplastic when there is a significant stromal component to the tumour. Most tumours of epithelial origin that occur in the gastrointestinal tract are relatively desmoplastic and pancreatic cancer is characteristically so (Cruickshank, 1986). It is this feature of PDAC that is investigated within the course of this thesis.

The cellular and matrix components of pancreatic cancer have been arbitrarily divided for descriptive purposes. Within the tumour they are intimately linked and occupy a close spatial arrangement.

Cells Found in Pancreatic Adenocarcinoma

- Cancer Cells: The malignant epithelium forms atypical, irregular glands, which may or may not secrete mucus. The epithelium varies between columnar and anaplastic cuboidal in structure. An adenosquamous or extremely anaplastic cellular pattern comprises approximately 10% of exocrine pancreatic tumours. Rarely tumours can arise in cysts (cystadenocarcinomas) or have acinar cell morphology (acinar cell carcinoma) (Cotran et al., 1989b).
- Myofibroblasts: Pancreatic stellate cells (PSC: described fully in Part 6) are recognised immunohistochemically as fibroblastoid cells that stain positively for α-smooth muscle actin (αSMA), in areas of interstitial stroma (Schuppan et al., 1999; Bachem et al., 1998; Apte et al., 1998). A few αSMA positive cells can be identified in the interlobular septa of the normal pancreas (Yen et al., 2002). In pancreatic cancer, however, intense αSMA staining throughout the desmoplastic reaction has been reported, suggesting the presence of numerous PSC (Yen et al., 2002; Bachem et al., 2000).
- Macrophages: Significantly more macrophages can be found in the stroma of pancreatic cancer than the normal pancreas (Linder et al., 2001; Emmrich et al., 1998).
- Other Immune cells: Pancreatic cancers contain a variable but increased number of CD4+ (50-90%) and CD8+ (5-20%) lymphocytes, compared to normal controls (Linder et al., 2001; Emmrich et al., 1998). Natural killer cells on the other hand were rarely seen (Linder et al., 2001).

Hence in comparison to the normal tissue, there are large numbers of myofibroblasts, with a variable inflammatory infiltrate in the stroma around the malignant glands.

Extracellular Matrix Content of the Desmoplastic Reaction

- Basement Membrane: In health a well defined, continuous basal lamina exists around pancreatic acini and ducts separating the epithelial cells from the interstitium (Mollenhauer et al., 1987). This is comprised predominantly of laminin and collagen type IV but collagen type V and heparan sulphate

proteoglycan (HSPG) can also be identified (Haglund et al., 1984; Imamura et al., 1995b; Lee et al., 1994; Ingber et al., 1985; Wang et al., 1994). A key feature of invasive malignancy of epithelial origin is loss of and invasion through the basement membrane and in pancreatic cancer the basement membrane is discontinuous and irregular in places (Stevens and Lowe, 1995a; Wang et al., 1994; Lee et al., 1994; Linder et al., 2001). The degree of basement membrane irregularity and loss correlates well with loss of differentiation of the tumour (Haglund et al., 1984; Mollenhauer et al., 1987). Furthermore, type IV collagen and laminin (normally confined to the basement membrane) can also be identified irregularly distributed throughout the interstitial stroma of pancreatic tumours (Haglund et al., 1984; Lee et al., 1994; Imamura et al., 1995b; Linder et al., 2001). HSPG is lost from the BM in tumours and this also correlates with differentiation, but is not seen in the tumour stroma (Wang et al., 1994). Certain laminin chains (γ_2) have been identified within the cytoplasm of pancreatic cancer cells (Takahashi et al., 2002). If such chains predominate in the cytoplasm, rather than the basement membrane, a poor prognosis ensues, although no clear mechanism underlying this association has been discovered (Takahashi et al., 2002).

- Interstitial Connective Tissue: In the normal pancreas, the supporting septa of connective tissue in interacinar spaces is comprised of collagen types I and III and fibronectin (Ingber et al., 1985). The larger interlobular ducts are additionally supported by sheets of fibrillar collagen (Mollenhauer et al., 1987). In pancreatic adenocarcinoma, the fine septal organisation is lost and replaced by large swathes of interstitial stroma, comprising predominantly of collagen types I and III (Mollenhauer et al., 1987; Linder et al., 2001). As previously stated, this is analogous to fibrous granulation tissue. Chemical analysis of whole tumour tissue reveals that there is a 3 fold increase in collagen content, over normal pancreas, with approximately 66% type I and 33% type III collagen (Imamura et al., 1995a; Ellenrieder et al., 2000; Gress et al., 1995). It is of interest that collagen in chronic pancreatitis (also characterised by fibrosis) is of a similar composition, suggesting the matrix has a common cellular origin (Imamura et al., 1995a). Fibronectin has been found distributed throughout the malignant stroma in a similar pattern to collagen I, however other authors have detected little more than in the normal pancreas (Mollenhauer et al., 1987; Linder et al., 2001). Proteoglycans (e.g. biglycan), are also over expressed in pancreatic cancer at RNA and protein level (Weber et al., 2001). Some extracellular matrix components of the tumour stroma are not detected in the

structure of the normal pancreas. Vitronectin and tenascin are both reported to be irregularly expressed in tumours, with a spatial tendency toward the cancer cells but are not found in the normal pancreas (Linder et al., 2001)

Hence the pathophysiological balance in pancreatic cancer favours the accumulation of stroma and this is associated with loss of normal tissue architecture and differentiation (Mollenhauer et al., 1987). The quality of extracellular matrix that malignant epithelial cells contact changes from basement membrane constituents to interstitial connective tissue, rich in fibrillar collagen.

4. Growth factors and cytokines in pancreatic cancer

Growth factors have been extensively researched in pancreatic cancer, as uncontrolled autocrine and paracrine growth factor stimulation appears to be very important in tumourigenesis (Schmielau et al., 1996). Many of these growth factors ligate cell surface receptors, increasing phosphorylation of cytoplasmic tyrosine kinases and tyrosine phosphatases and thereby stimulating cell division (Douziech et al., 1998). The over expression of growth factors and their receptors are considered to be of key importance in carcinogenesis (Stevens and Lowe, 1995a).

The epidermal growth factor (EGF) family (EGF, HB ECG, TGFα, amphiregulin) are all expressed at high levels by cancer cells in the tumour mass (Korc, 1998). There is also a high level of receptor expression by cancer cells and the whole family increases the proliferation rate of pancreatic cancer cells (Korc, 1998; Liehr et al., 1990).

The fibroblast growth factor (FGF) family of growth factors (1/aFGF, 2/bFGF, 3, 4, 5 and 7/KGF) are over expressed by pancreatic tumours and cell lines (Korc, 1998). They stimulate cancer cell proliferation (by activation of the MAPK pathway) and FGF-2 has also been shown to enhance invasion of pancreatic cancer cells (Korc, 1998; Douziech et al., 1998; Wagner et al., 1998b).

The insulin-like growth factor (IGF) family (IGF 1, 2 and insulin) are important in cell cycle progression and they too are thought to act as auto/paracrine growth factors in pancreatic cancer cells (Korc, 1998; Douziech et al., 1998). Insulin is not over expressed in pancreatic adenocarcinoma, but cells of the pancreas may be exposed to high local concentrations (via locoregional blood flow) and it too acts as a growth factor (Liehr et al., 1990; Korc, 1998).

Transforming growth factor β (TGFβ) is over expressed in pancreatic cancer but tends to inhibit proliferation of pancreatic cancer cells (Giehl et al., 2000; Friess et al., 1993). The down stream targets such as CTGF may, however, play an important role in cancer cell growth (Korc, 1998). The key roles that TGFβ may play in the regulation of extracellular matrix in pancreatic tumourigenesis are discussed further in Part 6.

Platelet derived growth factor (PDGF) and its receptors are over expressed in pancreatic cancer and stromal cells (Kalthoff et al., 1991; Ebert et al., 1995). This expression is inducible by cytokines like tumour necrosis factor α (TNFα) (Kalthoff et al., 1991).

Connective tissue growth factor (CTGF) has been demonstrated in numerous pancreatic cancer cell lines but in pancreatic cancer tissue it is mainly located to stromal cells, where it may act as an important mitogen (Wenger et al., 1999). EGF and TGFα have been shown to up regulate CTGF expression by cancer cells (Wenger et al., 1999).

Vascular endothelial growth factor (VEGF) is an important endothelial growth factor and it too is expressed by pancreatic cancer cells (Jaster et al., 2002). Its importance in pancreatic tumourigenesis has been demonstrated using blocking antibodies in xenograft models, which inhibit angiogenesis and consequently tumour growth (Jaster et al., 2002).

In common with other malignancies pancreatic cancer is characterised by abundant growth factor and growth factor receptor expression.

5. Gastrointestinal hormones in pancreatic cancer

As well as the traditional growth factors, cells of the exocrine pancreas are under local hormonal control by the gut. Gastrin, bombesin, cholecystokinin (CCK) and caerulein (CCK analogue) have been shown to stimulate proliferation of pancreatic cancer cells (Douziech et al., 1998; Lewis et al., 1998). Other studies of bombesin and CCK, however, have shown that these hormones exert minor and more variable effects on pancreatic cancer cell growth. This is also true of vasoactive intestinal peptide (VIP) and somatostatin (Liehr et al., 1990; Douziech et al., 1998;

Lewis et al., 1998). The conflicting reports in the literature probably represent the complex nature in which these hormones act and interact in vivo (Lewis et al., 1998).
Gastrointestinal hormones, therefore, play an inconsistent role in pancreatic cancer.

6. MMP and TIMP expression in pancreatic cancer

MMPs are thought to play an important role in pancreatic tumour biology (Jones et al., 1999). Table 1.2 summarises studies examining MMP and TIMP expression in pancreatic cancer (Bramhall et al., 1997; Bramhall et al., 1996; Gress et al., 1995; Ito et al., 1999; Crawford et al., 2002). Normal pancreas controls were used in these studies and in general there was little or no expression of the MMPs studied, although TIMP-1 mRNA was detected in the normal controls of 2 studies, albeit at comparatively low levels (Bramhall et al., 1997; Gress et al., 1995). These studies provide good evidence that MMPs and TIMPs are expressed at high levels in pancreatic cancer (in tumour and stromal cells) and they seem to play numerous roles in the malignant phenotype.

MMP-7 (matrilysin) may play a key role in pancreatic carcinogensis. In animal models of acinar-ductal metaplasia, it accumulates in the metaplastic epithelium. MMP-7 cleaves Fas ligand, which now soluble, induces acinar cell apoptosis, promoting ductal metaplasia. MMP-7 or Fas ligand knockout mice do not develop metaplasia, supporting the hypothesis (Crawford et al., 2002).

Gelatinases (MMP-2 and 9) degrade basement membrane components. In pancreatic cancer their over expression correlates with the degree of desmoplasia, venous invasion and worse prognosis (Ellenrieder et al., 2000; Matsuyama et al., 2002; Nagakawa et al., 2002). These descriptive studies have been supplemented by studying the effect of whole tumour homogenates in gelatin zymography (Koshiba et al., 1998). This demonstrated latent MMP 2 and 9 in all normal and pancreatic cancer tissues (Koshiba et al., 1998). However, the active form of MMP-2 was present in 100% of cancer tissues compared to 30% of normal tissues (with MMP-9 activity similar between tissues) (Koshiba et al., 1998). Therefore abundant MMP expression correlates with enhanced activity in pancreatic cancer.
Furthermore, the high ratio of active to inactive MMP-2 correlated well early locally recurrent disease after pancreatic resection (Koshiba et al., 1998).

Functional studies of MMPs and TIMPs in pancreatic cancer have been undertaken using pancreatic cancer cell lines in *in vitro* and *in vivo* models. Constitutive expression of active MMPs by pancreatic cancer cells is inconsistent (Bramhall et al., 1997). It can be up regulated by orthotopic inoculation in nude mouse models, suggesting that MMP expression/activity may be under represented *in vitro* (Haq et al., 2000). MMP expression is induced in pancreatic cancer cell lines by a variety of substances including collagen I, TGFβ, 12-O-tetradecanoylphorbol-13-acetate and concanavalin and this is accompanied by enhanced invasive capacity (Yang et al., 2001; Teraoka et al., 2001).

Interaction between cancer and stromal cells may modulate MMP expression. Stromal cells may up-regulate MMP-9 expression by pancreatic cancer cells via thombospondin-1, which appears to originate from the stromal elements of the tumour (Qian et al., 2001). There is also evidence that cancer cell MT1-MMP is involved the binding and activation of stromally secreted MMP-2 in pancreatic cancer (Ellenrieder et al., 2000). MT1-MMP is increasingly recognised as a key player in the malignant phenotype in a variety of tumours (Hotary et al., 2003).

		Northern Blotting	Immunostaining		In-SItu Hybridisation	
		Whole Tissue	Malignant Cells	Stromal Cells	Malignant Cells	Stromal Cells
MMP	1	Not Detected	+++			
	2	+++	+++	++	+++	+++
	3	+	+++	+		
	7	+++				
	9	+++			+++	++
	10	Not Detected				
	11	++				
	MT1	++			++	+
TIMP	1	+++	+++	+	+++	+++
	2	++			+++	+++

Table 1.2
Summary of major studies of MMP expression in post mortem pancreatic cancer specimens (Bramhall et al., 1997; Bramhall, 1997; Gress et al., 1995; Ito et al., 1999; Ellenrieder et al., 2000)
+++ intense staining/>66% samples stain positively
++ intermediate staining/33-66% samples stain positively
+ weak staining/<33% samples stain positively
[where blank: no published data]

The important role of MMPs in malignancy can also be demonstrated by inhibiting their activity using synthetic MMP inhibitors. Cell lines with higher constitutive MMP-2 and/or 9 expression readily invade Matrigel (AsPC-1, Panc-1 and Capan-1) but this can be arrested with MMP inhibition (Jimenez et al., 2000; Zervos et al., 1999a; Ellenrieder et al., 2000). Similarly, if these cells are injected into nude mice, MMP inhibition can significantly reduce hepatic metastasis and tumour burden (Jimenez et al., 2000). Other studies have also found that MMP inhibitors slow the growth of tumours and correlate this to reduced MMP-2 activity, underlining the key importance of MMP-2 in pancreatic cancer (Zervos et al., 1999b).

MMPs are also likely to be important in angiogenesis in pancreatic tumours but there is no experimental evidence to confirm this as yet. Hence, in vitro MMPs would seem to play a key role in the malignant phenotype, yet the use of MMP inhibitors in patients with pancreatic cancer has not been shown to improve the prognosis, although proponents believe that this is due to a failure of study design (Bloomston et al., 2002a). This aside, such findings bear testament to the complex nature of MMP function in tumours outlined in Part 4.

Endogenous MMP inhibitors appear to be expressed by stromal and malignant cells alike in pancreatic cancer, in the form of TIMP-1 and to a lesser extent TIMP-2 (Bramhall et al., 1997). Over expression of both TIMP-1 and 2 after adenoviral transfection of pancreatic cancer cell lines reduces invasion *in vitro* (Rigg and Lemoine, 2001). If such transfection is undertaken *in vivo* (at a very early stage after tumour inoculation in nude mice), reduced tumour burden, ascites and prolonged survival is seen (Rigg and Lemoine, 2001). The therapeutic potential of this remains questionable because it had little or no effect after the tumours had time to implant (Rigg and Lemoine, 2001). Other studies confirm the apparently beneficial effect of TIMP-1 transfection, with reduced tumour implantation, growth, metastasis and angiogenesis after orthotopic implantation (Bloomston et al., 2002b).

It is not only TIMPs that inhibit MMPs. mRNA for α2-macroglobulin has also been demonstrated in stromal cells of pancreatic cancer and this acts as a rather non-specific protease inhibitor (Iacobuzio-Donahue et al., 2002a). Retinoic acid reduces MMP expression by pancreatic cancer cells (von Marschall et al., 1998). One may speculate about its relevance in pancreatic cancer, in that quiescent stellate cells characteristically store abundant retinoids in the pancreas (Bachem et al., 1998).

The marked desmoplastic reaction seen in pancreatic cancer does suggest the overall balance favours matrix accumulation rather than degradation in pancreatic cancer, perhaps reflecting consistent TIMP expression and inconsistent MMP expression.

7. Integrin expression and adhesion to extracellular matrix in pancreatic cancer

Integrins are a large family of homologous transmembrane receptors that allow cells to bind to extracellular matrix (Alberts et al., 1994). There are other classes of cell adhesion receptors including cadherins and members of the immunoglobulin/selectin families but integrins are particularly relevant to this work because they act as important paths of communication between the extracellular matrix and the nucleus of a cell (van der and Sonnenberg, 2001; Schwartz, 2001).

Integrins are comprised of a dimer of α and β subunits (Alberts et al., 1994). 24 α subunits and 9 β subunits have been identified and they form a variety of combinations (van der and Sonnenberg, 2001). Those found in pancreatic cancer (to date) are summarised from a number of studies in table 1.3. In general, the integrins expressed in pancreatic cancers are the same as those found in the normal pancreas (Hall et al., 1991). The principal difference in expression pattern is the more diffuse integrin expression in malignant cells compared to the polarised expression seen in normal pancreatic epithelium (Linder et al., 2001; Weinel et al., 1992). This may reflect the loss of spatial arrangement of tumour cells, which leaves them armed with means to attach to extracellular matrix during invasion and metastasis (Weinel et al., 1992). Integrin expression patterns amongst pancreatic cancer cell lines do not correlate with the degree of differentiation (Lohr et al., 1996).

Integrins are quite promiscuous with respect to ligand binding and have been shown ligated to MMPs and growth factors in addition to ECM (van der and Sonnenberg, 2001). Signals are transmitted through the cell membrane where the intracellular integrin domain interacts with a whole host of cytoplasmic proteins including cytoskeletal proteins (e.g. actin/talin), adaptor and signalling proteins (e.g. integrin-cytoplasmic domain associated protein (ICAP-1)), protein kinases (e.g. focal adhesion kinase (FAK)), chaperone proteins and transcription factors (van der and Sonnenberg, 2001). Through these interactions, integrin signalling has been

coined as 'outside in' and 'inside out', in recognition of the dynamic role that they play in cellular communication (Schwartz, 2001). Other important interactions occur through communications with other cell membrane receptors including growth factor receptors (e.g. EGFr/PDGFr) and immunoglobulin proteins (e.g. EMMPRIN). There is emerging evidence that integrins can collaborate with intracellular signalling pathways initiated by growth factors such as VEGF, PDGF and IGF (Zucker et al., 2001). Therefore the response of cells to soluble growth factors is critically dependent on the pericellular extracellular matrix.

The potential role of integrins in the pathophysiology of pancreatic cancer has not been widely investigated. It is known that pancreatic cancer cells bind to collagen, fibronectin and laminin via $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ integrins respectively, which represents the normal situation in non-malignant epithelium (refer to appendix 1a for table listing integrins and ECM ligands) (Arao et al., 2000; Lohr et al., 1996). Pancreatic cancer cell lines adhere well to fibrillar collagen, laminin and fibronectin in vitro and adhesion to a matrix can lead to up-regulation of the relevant integrin receptor (McIntyre et al., 1981; Haberern and Kupchik, 1985; Rosewicz et al., 1997; Menke et al., 2001). Adhesion to type IV collagen is enhanced by fibronectin (contained in FCS) or by the addition of laminin (McIntyre et al., 1981; Haberern and Kupchik, 1985). The growth factors EGF, TGFβ and Il-1α (which foetal calf serum potentially contains) are said to modulate adhesion to type I collagen and fibronectin (Stefani et al., 1999). It is therefore important to recognise that a variety of factors can influence cell adhesion.

β_1 integrins have variable constitutive activity, although the mechanisms underlying this are not understood (Arao et al., 2000). Retinoids, for example, selectively reduce the function of $\alpha_6\beta_1$ integrin of pancreatic cancer cells, inhibiting binding to laminin and fibronectin (Rosewicz et al., 1997). Despite investigation of this phenomenon there was no obvious change in $\alpha_6\beta_1$ expression (Rosewicz et al., 1997). The constitutive activity of β_1 integrins in pancreatic cancer cell lines correlates well with their ability to invade Matrigel (Arao et al., 2000). Furthermore β_1 integrin blocking antibodies substantially reduces tumour formation after venous injection of pancreatic cancer cells in nude mice (Vogelmann et al., 1999). A similar effect was seen with blocking antibodies to the α_6 integrin subunit (Vogelmann et al., 1999). The therapeutic angle has been investigated using lansoprazole that binds to β_1 integrin, causing a conformational change (Ohta et al., 1999). It reduces

cell adhesion in vitro and tumour growth in vivo (Ohta et al., 1999). β_1/β_4 integrins have also been implicated in mediating differentiation of pancreatic cancer cells in vitro (Stagge et al., 2001).

Integrin Subunit		Present	Comment
β	1	In all histological sections and cell lines tested	Polarity of integrin expression lost with loss of basement membrane, positive staining in the stromal cells
	3	Found in 2 cell lines, but not in histological sections	No staining for β3 integrin in either the normal or malignant pancreas
	4	Detected in cell 2 cell lines but not in histological sections	Acinar and ductal cells stain for β-4 integrin in the normal pancreas
α	1	Detected in cell lines and histological sections	Some studies did not detect α-1 subunit at all
	2	Universally present	More intense staining in malignant than control sections, localised to the basement membrane if present
	3	Universally present	More intense staining in malignant than control sections, localised to the basement membrane if present
	4	Detected in one study of histological sections	Other studies fail to detect it in histological specimens or cell lines
	5	Found in cell lines but not detected in histological sections	
	6	Universally present in all but one study	
	v	Found in histological sections and cell lines	Pattern of expression correlated well with vitronectin and tenascin distribution

Table 1.3
Integrin expression in pancreatic cancer
Compiled from the following studies (Linder et al., 2001; Arao et al., 2000; Vogelmann et al., 1999; Hall et al., 1991; Shimoyama et al., 1995)

Cadherins also seem to be important in the malignant phenotype. E-cadherin is the major receptor in mediating cell to cell adhesion in epithelia (Alberts et al., 1994). E-cadherin expression is reduced in metastatic cell lines (Jimi et al., 1998). Fibrillar collagens have been shown to reduce E-cadherin expression levels in pancreatic cancer cells, which may promote the invasive/metastatic phenotype (Menke et al., 2001). Furthermore a study of post mortem specimens has linked reduced E-

cadherin expression with a worse prognosis in pancreatic cancer (Kuniyasu et al., 1999).

Hence matrix receptors (particularly integrins) are important in mediating phenotypic changes in pancreatic cancer cells.

8. Origin and effects of extracellular matrix in pancreatic cancer

Transcripts for most constituents of the extracellular matrix have been demonstrated in pancreatic cancer cells but at a relatively lower level than fibroblasts (Lohr et al., 1994). Laminin is also secreted by cancer cells *in* vitro (Tani et al., 1997; Haberern and Kupchik, 1985). However, much of the cancer literature suggests that the role of matrix synthesis in the desmoplastic reaction is fulfilled by myofibroblasts (Ohtani et al., 1992). As stated there are numerous myofibroblasts in pancreatic cancer and there is now some evidence that they synthesise extracellular matrix in response to pancreatic cancer cells (Yen et al., 2002; Bachem et al., 2000).

One of the central interests of this research was to determine if the change in quality of the extracellular matrix found in pancreatic cancer has an effect on the way tumour cells behave. Some information is available from a number of disparate studies that have previously addressed this question in pancreatic cancer. The degree of cellular differentiation affects the way cells behave. Poorly differentiated cells form only loose aggregates of cells on Matrigel and collagen I gel (Yamanari et al., 1994). In contrast, well differentiated pancreatic cancer cells actually form glandular structures on both substrata and those cultured on Matrigel even formed basal lamina (Yamanari et al., 1994). Matrigel is a tumour matrix secreted by Engelbreth-Holm-Swarm mouse sarcoma containing type IV collagen and laminin (Wisdom, Jr. et al., 1992). Culture of pancreatic cancer cells on a laminin-nidogen substratum (also derived from Matrigel) allowed sub-culture of a set of cells that had apparently re-differentiated, regaining ductal phenotype (Paddenberg et al., 1998). This was characterised by cellular polarity and the development of duct like luminal spaces as well as a reduction in protease secretion (plasminogen activators and cathepsin), all suggestive of re-differentiation (Paddenberg et al., 1998). Other evidence for laminin being inhibitory to the malignant phenotype was reflected in the reduced proliferation rate of cancer cells cultured on laminin, compared to those on type I collagen and fibronectin (Mollenhauer et al., 1987). Fibronectin and laminin have also been shown to reduce the basal apoptosis rate, with respect to tissue culture plastic in cultured pancreatic cancer cells (Vaquero et al., 2000). This

was blocked by PI3K inhibition, which is known to be a downstream target of integrin signalling (Vaquero et al., 2000; Schwartz and Assoian, 2001). Hence there is some evidence that extracellular matrix may modulate proliferation and apoptosis in pancreatic cancer.

The other major component of basement membrane, type IV collagen has also been found to reduce the proliferation rate of a number of cancer cell lines, including a pancreatic line (Shahan et al., 1999a). This was attributed to increased levels of cAMP in cells cultured on collagen IV (Shahan et al., 1999a). Evidence is emerging that suggests fibrillar collagen may also affect proliferation and invasion of pancreatic cancer cells (Menke et al., 2001). Neither fibrillar nor basement membrane collagen effects the basal apoptotic rate of pancreatic cancer cells (Vaquero et al., 2000).

Another component of the desmoplastic reaction, bigycan has a dose dependant anti-proliferative effect on pancreatic cancer cells (Weber et al., 2001). Cell growth was arrested in G_1 by a reduction in cyclin A and PCNA, with a concomitant increase in p27 (Weber et al., 2001).

These findings indicate that extracellular matrix is important in the phenotype of pancreatic cancer.

Part 6
Pancreatic Stellate Cells

1. Introduction

The myofibroblastic phenotype is the common phenotype acquired by fibroblasts, smooth muscle cells and also 'stellate' cells, subjected to the appropriate stimulus (shown in figure 1.5) (Powell, 2000; Sappino et al., 1990; Ronnov-Jessen and Petersen, 1993a). Rather confusingly myofibroblasts seen in tumour stroma are also called peritumoural fibroblasts, reactive stroma and carcinoma-associated fibroblasts (CAFs)(Tlsty and Hein, 2001). Nomenclature aside, it is cells with a myofibroblastic phenotype that appear in tumour stroma and orchestrate the stromal 'repair' response discussed above (Sappino et al., 1990). Current evidence suggests that the transdifferentiation of pancreatic stellate cells is responsible for the myofibroblast population in the stroma of pancreatic adenocarcinoma (Apte et al., 1998; Bachem et al., 1998; Yen et al., 2002).

2. History

Stellate cells were first identified in the liver, where they have been extensively researched in the context of liver cirrhosis (Berk and Friedman, 2001). Cirrhosis is the common response of the liver to a number of injurious stimuli resulting in progressive fibrosis that results in liver failure. This process appears to be partially reversible with removal of the aetiological factor (Iredale, 2001). More recently pancreatic stellate cells (PSCs) have been identified and characterised (Apte et al., 1998; Bachem et al., 1998). They are implicated in the fibrosis that typifies chronic pancreatitis and it is in this context that they have been most extensively studied. Current evidence suggests that the behaviour of PSCs and hepatic stellate cells (HSC) is very similar (Bachem et al., 2002).

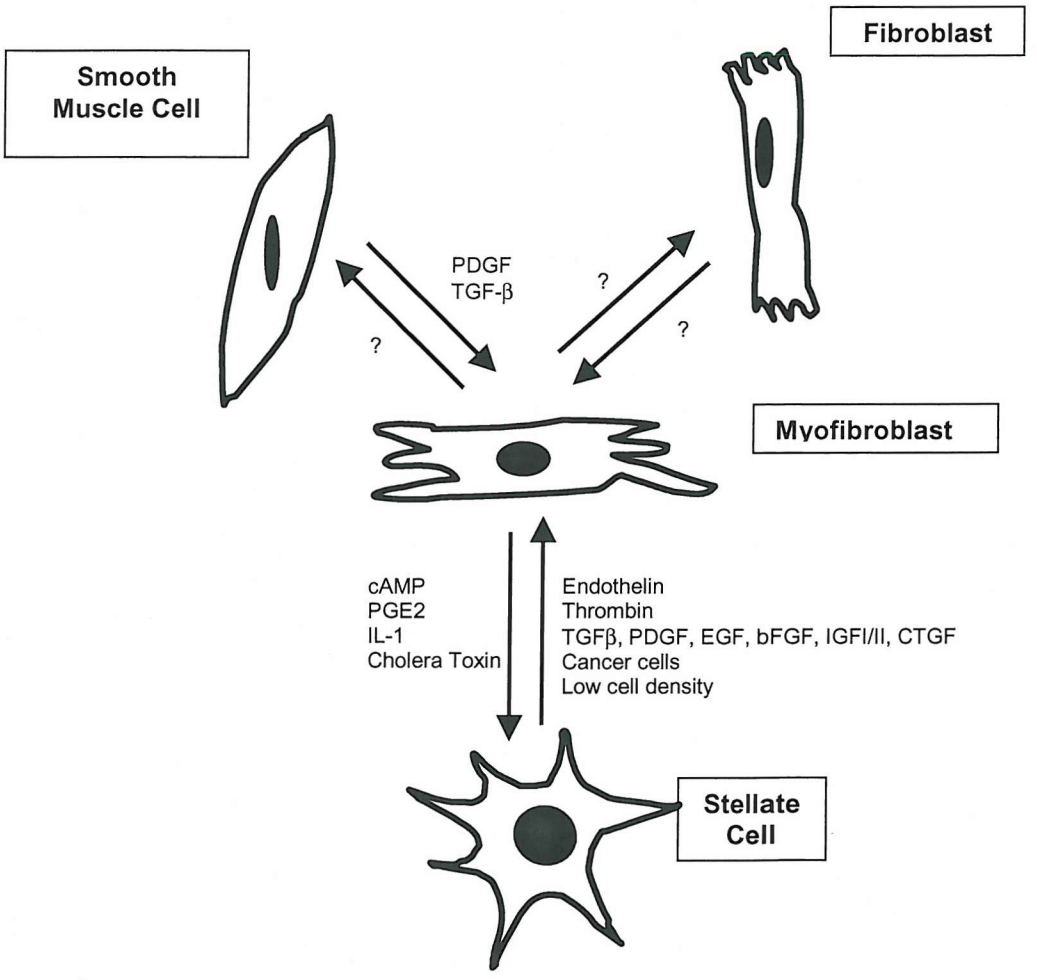


Figure 1.5
Diagram showing cells with potential for myofibroblastic transdifferentiation
(Powell, 2000)

3. Stellate cell activation and growth

In health pancreatic stellate cells exist in 'quiescent' phenotype (Apte et al., 1998; Bachem et al., 1998; Schuppan et al., 1999). This is characterised by retinoid

storage and expression of desmin and glial fibrillary acid protein (GFAP) (Bachem et al., 1998; Apte et al., 1998). They are found in interacinar and interlobular spaces, with their long cytoplasmic processes encircling adjacent pancreatic acinar cells (Apte et al., 1998; Bachem et al., 1998).

It is when stellate cells become activated in a disease process and adopt a myofibroblastic phenotype that they play a central role in the pathophysiology of the disease (Haber et al., 1999; Yokota et al., 2002). The myofibroblastic phenotype is characterised by loss of retinoid droplets, αSMA expression and secretion of extracellular matrix (Bachem et al., 1998; Apte et al., 1998).

The mechanisms by which stellate cells become 'activated' or transdifferentiate has been well studied and characterised, particularly in the context of liver fibrosis (Gressner, 1995). It is difficult and somewhat arbitrary to separate activation of the myofibroblastic phenotype from the subsequent proliferation and enhanced extracellular matrix secretion. Transforming growth factor-beta (TGFβ) seems to play a key role in stellate cell transdifferentiation (Desmouliere et al., 1993; Ronnov-Jessen and Petersen, 1993b; Powell, 2000; Bedossa and Paradis, 1995). It has been shown to accelerate the development of αSMA expression in cultured pancreatic stellate cells (Apte et al., 1999; Schneider et al., 2001). TNFα, Il-1, Il-6 and Il-10 also increase αSMA expression and may be involved in the activation process as well (Mews et al., 2002). *In vivo* mast cell degranulation is associated with activated PSC in chronic pancreatitis but whether this is a causal association remains unknown (Zimnoch et al., 2002).

PSC proliferation is stimulated by PDGF-AB and bFGF, both of which act as powerful mitogens (Schneider et al., 2001; Luttenberger et al., 2000; Apte et al., 1999). There is conflicting evidence as to whether TNFα has a mitogenic effect but TGFα, IGF-1, IGF-2, interleukin-1 (Il-1), Il-6 and Il-10 do not increase the rate of PSC proliferation (Mews et al., 2002; Schneider et al., 2001). CTGF has been shown to accelerate HSC proliferation but this has not been examined in PSC biology (Paradis et al., 2002). Although it stimulates matrix synthesis, TGFβ inhibits proliferation (Schneider et al., 2001; Shek et al., 2002; Kruse et al., 2000).

Circumstantial evidence would suggest that cancer cells may be capable of activating stellate cells because they over express many of these factors.

4. Matrix synthesis and degradation

Myofibroblasts secrete ECM, which is important in wound healing (Stevens and Lowe, 1995c). With a persistent/abnormal stimulation of myofibroblasts, however, matrix accumulates, leading to abundant fibrous stroma, which is often largely responsible for the clinical syndrome of the disease (Braganza, 1996; Iredale, 2001). The control of matrix secretion by activated pancreatic stellate cells has been studied and unsurprisingly it has many similarities with matrix secretion by fibroblast/smooth muscle derived myofibroblasts (Powell, 2000). A number of growth factors and cytokines seem to play an important role.

Table 1.4 shows the soluble factors that have been shown to regulate matrix synthesis by pancreatic stellate cells. CTGF expression correlates with the degree of fibrosis in chronic pancreatitis but its functional effect on PSCs has not been tested (di Mola et al., 1999). It has been shown that CTGF increases collagen I expression in HSCs (Paradis et al., 2002). As well as secreting matrix, PSC also regulate matrix turnover by secreting MMPs (2, 3, 9, 13 and MT-1 MMP) and TIMP-1 and 2 (Shek et al., 2002; Phillips et al., 2003). Increased MMP expression occurs after PSC activation or stimulation with Il-6 (Phillips et al., 2003).

	Matrix		
	Collagen I	**Collagen III**	**Fibronectin**
Growth Factors increasing matrix synthesis	bFGF TGFβ PDGF TGFα TNFα platelet lysate Il-10	bFGF TGFβ PDGF AB	bFGF TGFβ PDGF AB TGFα platelet lysate
Growth Factors with no effect	Il-1 Il-6		TNFα IGF I/II

Table 1.4
Soluble factors that regulate pancreatic stellate cell matrix synthesis
(Schneider et al., 2001; Mews et al., 2002; Shek et al., 2002; Luttenberger et al., 2000; Apte et al., 1999)

5. Special importance of TGFβ in pancreatic fibrosis

TGFβ has been widely studied and in common with many organs, its over expression appears to be central to the development of fibrosis in the pancreas (Muller-Pillasch et al., 1999). In animal pancreatitis models, $TGF\beta_1$ gene expression

greatly exceeds that in corresponding normal pancreas and correlates well with increased ECM gene expression (Su et al., 2000; Muller-Pillasch et al., 1999). Studies of resected chronic pancreatitis specimens show increased $TGF\beta_1$/TGFβ type II receptor expression (di Mola et al., 1999).

It is likely that the desmoplastic reaction in pancreatic cancer has a common cellular origin to the fibrotic reaction of chronic pancreatitis and circumstantial evidence implicates $TGF\beta_1$ in its pathogenesis (Imamura et al., 1995a). $TGF\beta_1$ is over expressed by pancreatic cancer cells and this phenomenon strongly correlates to reduced survival (Friess et al., 1993; Satoh et al., 1998). Furthermore, macrophages and myofibroblasts stain strongly for TGFβ in the stroma (Friedman and Arthur, 1989; Bachem et al., 1992; Kruse et al., 2000; Satoh et al., 1998). Functional studies show that $TGF\beta_1$ transfected pancreatic cancer cells will increase fibroblast growth and collagen synthesis and also enhance desmoplasia in orthotopic tumour implants (Lohr et al., 2001).

Interestingly the tumour cells have been reported to have a reduced expression of TGFβ receptors, particularly types II and III and it has been postulated that this may allow the cancer cells to escape the anti-proliferative effects of TGFβ (Venkatasubbarao et al., 2000; Freeman et al., 1995) (although one other study has demonstrated TGFβ receptors in pancreatic cancer (Satoh et al., 1998)). Mutation of Smads (cytoplasmic proteins important in TGFβ signal transduction) is another mechanism by which pancreatic cancer cells may escape growth regulation by TGFβ (Weber et al., 2001; Simeone et al., 2000). A reduction in TGFβ receptor expression is a common finding in malignancy; it has also been reported in gastric, prostate and T-cell malignancies (Korc, 1998).

Part 7

Evidence for interaction between cancer cells and desmoplastic reaction influencing the phenotype of cancer

1. Introduction

Most neoplastic tumours contain stromal elements. The stromal component of tumours is more akin to the granulation tissue found in healing wounds, than connective tissue found in normal tissue, the obvious difference being that it contains malignant cells (Dvorak, 1986). Much cancer research in the past

concentrated on the cancer cells themselves but it is now accepted that the supporting stroma within tumours plays an important biological role in neoplastic behaviour and growth (Seljelid et al., 1999; Tuxhorn et al., 2001; Tlsty and Hein, 2001; Noel and Foidart, 1998; De and Mareel, 2002; Ingber, 2002). The phenotypic effect that stroma exerts on tumour behaviour varies with tumour type and consequently it remains debatable whether it represents a beneficial immunological response or an inadvertent catalyst to tumour development (Seljelid et al., 1999). Malignant cells are often 'anchorage' independent, yet still they respond to the extracellular matrix (Radisky et al., 2002). The desmoplastic reaction in relation to pancreatic cancer has been described in Part 5 but more can be learned about the effects of the stromal compartment on tumour behaviour from other cancer research.

2. Stromal cells in carcinogenesis and tumourigenesis

Myofibroblasts are found in the stroma of most desmoplastic tumours, including pancreatic, colonic, breast and prostate cancers (Yen et al., 2002; Martin et al., 1996; Noel and Foidart, 1998; Tuxhorn et al., 2001). As previously discussed, it would appear that the quiescent fibroblast or stellate cell must first acquire its functioning myofibroblastic phenotype before playing a role in tumourigenesis. Their initial activation in cancer is likely to be due to paracrine stimulation by cytokines, which may be secreted by cancer cells (Schurch, 1999; Ooi, 1999; Valenti et al., 2001). Evidence for this is seen in increased numbers of myofibroblasts in close vicinity to dysplastic and malignant but non-invasive epithelia (in-situ carcinoma) and adenomatous polyps (Schurch, 1999; Adegboyega et al., 2002). However, they become far more numerous once malignant invasion through the basement membrane has taken place and tumours develop (Yen et al., 2002; Martin et al., 1996; Noel and Foidart, 1998; Tuxhorn et al., 2001). Hence the activation of myofibroblasts in malignancy would appear to be a progressive event, which speeds up as malignant invasion of the interstitial matrix occurs, rather than this event being a pre-requisite for myofibroblast activation in itself (Schurch, 1999). Indeed myofibroblasts may help facilitate invasion of malignant cells through the basement membrane (Zucker et al., 2001).

There is further evidence for the role of myofibroblasts in tumourigenesis [reviewed in (Tlsty and Hein, 2001)]. Immortalised 'benign' prostatic epithelial cells when grafted with myofibroblasts produced tumours 200 times the size of prostatic epithelial cells alone (Olumi et al., 1999). Co-injection of fibroblasts with breast

cancer cells significantly increased the likelihood of tumours forming and also growing to a larger size (Noel and Foidart, 1998). In another model of chemically induced hepatocellular carcinoma, as hepatocytes became dysplastic, activated hepatic stellate cells appeared closely associated with and among the hepatocytes (Johnson et al., 1998). These became more abundant with time and although clearly a host response, it was not clear if this represented a protective host response or an innocent usurping of resident stellate cells in tumourigenesis (Johnson et al., 1998). Hepatic stellate cells have also been implicated in angiogenesis in malignant melanoma metastasis by secreting VEGF (Olaso et al., 2003). It has also been postulated that the mechanical properties of tumour associated myofibroblasts/matrix prevent immune cells from contacting malignant cells, thereby preventing destruction of the cancer cells (Lieubeau et al., 1999).

Evidence for the role of myofibroblasts in the malignancy, suggests a potentially deleterious role in a broad variety of cancers.

3. Evidence for myofibroblasts synthesising extracellular matrix in cancer

Co-culture of anaplastic thyroid carcinoma cells and fibroblasts leads to up regulation of procollagen I mRNA in fibroblasts (Dahlman et al., 2002). This was found to be modulated by TGFβ and PDGF (Dahlman et al., 2002). Using in-situ hybridisation and immunoelectron microscopy, pro α1 collagen transcripts were identified in stromal cells but not cancer cells in 26 gastric and colorectal adenocarcinomas (Ohtani et al., 1992). Collagen synthesis by cells derived from colonic adenocarcinomas were studied in vitro and found collagen to be synthesised by the fibroblastic cells, with negligible amounts originating from the tumour cells (Turnay et al., 1989). Pilot studies in pancreatic cancer would support the concept that ECM originates from the stromal cells (Bachem et al., 2000).

4. Mechanisms by which extracellular matrix effects tumour behaviour

Extracellular matrix has been shown to effect changes in cancer cell behaviour. It can influence tumour cell number by changes in proliferation and apoptosis. Some clue to the mechanisms behind this can be gained from other tumours. Type I collagen gives prostate cancer cells a proliferative advantage compared to fibronectin and plastic (Kiefer and Farach-Carson, 2001) This was mediated by PI3K and MAPK pathways and increased cyclin D_1 expression (Kiefer and Farach-Carson, 2001).

It is not known whether the extracellular matrix in pancreatic cancer influences cellular resistance to chemotherapy but there is a growing body of evidence that this is the case in other malignancies. Non-adherent small cell lung cancer cells exhibit a dramatic reduction in apoptosis to a variety of chemotherapeutic agents in the presence of fibronectin, laminin and collagen type IV (Sethi et al., 1999). This effect was mediated by β_1 integrins, which led to increased cytoplasmic protein tyrosine kinase activity, protecting against activation of caspases and therefore apoptosis (Sethi et al., 1999). Extracellular matrix derived from stromal cells and purified matrices (collagen I/fibronectin) had a variable effect on susceptibility of colorectal cancer cell lines to chemotherapy induced apoptosis (Kouniavsky et al., 2002). Vitronectin, however, reduces chemotherapeutically induced apoptosis in glioma cells through induction of changes in bcl-2/bcl-X_L:bax ratio, suggesting there may be multiple mechanisms at work (Uhm et al., 1999).

Type IV collagen can cause an apparently diverse range of effects on cancer cells. It inhibits tumour cell proliferation, angiogenesis and reduces migration (Petitclerc et al., 2000; Maeshima et al., 2000; Pasco et al., 2000a). It also reduces MT1-MMP expression and MMP-2 activity (Martinella-Catusse et al., 2001; Pasco et al., 2000a). These effects have been attributed to non-collagenous domains of type IV collagen that bind to $\alpha_v\beta_3$ integrin leading to increased cellular cAMP and phosphorylation of FAK and PI3K (Shahan et al., 1999b; Pasco et al., 2000b).

In bile duct malignancies, however, type IV collagen supports tumour growth (Chen et al., 2001). It has also been shown to increase MMP-2 and 9 expression in neuroblastoma cells (although this was accompanied by a rise in TIMP expression, preventing simple conclusions being drawn)(Tzinia et al., 2002). The mechanism of action was attributed to ligation of $\alpha_3\beta_1$ rather than $\alpha_v\beta_3$ which may explain the difference (Tzinia et al., 2002). Matrigel (containing collagen type IV) enhanced tumour formation after heterotopic injection of a variety of tumour cells in nude mice (Noel and Foidart, 1998). The reasons cited for these observations included enhanced angiogenesis, stimulation of tumour protease expression but perhaps most likely is that Matrigel contains growth factors (EGF, IGF-1, PDGF, TGFβ and bFGF)(Noel and Foidart, 1998). Collagen IV (and laminin) also increased invasion of renal cancer cells in a micro-chemotaxis chamber via β_1 integrin dependent pathways (Brenner et al., 2000). Hence the effect of type IV collagen on phenotype may vary between cancers.

With reference to extracellular matrix, it would appear cancer cells may be 'anchorage independent' but they are not 'anchorage insensitive' (Declerck, 2000).

5. Role of stroma in tumour cell invasion

Myofibroblasts may play a role in cancer cell invasion. As described earlier pancreatic cancer cells are themselves capable of synthesising a range of MMPs and TIMPs but it is increasingly recognised that stromal cells also secrete MMPs in tumours (Hornebeck et al., 2002). MMPs may degrade basement membrane facilitating invasion, releasing growth factors or revealing cryptic extracellular matrix sites (that may promote cell proliferation/ migration) (Egeblad and Werb, 2002; Liotta and Kohn, 2001). MMPs also cleave E-cadherin permitting detachment of cells and invasion through the interstitial stroma (Egeblad and Werb, 2002).

The classic example of cancer/stromal cooperation is MMP-2, which is perhaps the best studied MMP synthesised by stromal cells (Egeblad and Werb, 2002; Declerck, 2000). MMP-2 can be found in the rough endoplasmic reticulum of stromal cells (suggesting active synthesis) but not in carcinoma cells (Ohtani, 1998). In vitro studies have found that breast cancer cells had very little constitutive MMP-2 expression relative to myofibroblasts (Singer et al., 2002). Zymography revealed that transfer of supernatant from the breast cancer cells increased MMP-2 activity in fibroblasts and direct co-culture of the cells also induced stromal cell MMP-9 expression (Singer et al., 2002).

A possible mechanistic explanation for this is through a member of the immunoglobulin superfamily: extracellular matrix metalloproteinase inducer (EMMPRIN) (Zucker et al., 2000). This has been found to be over expressed on the surface of a number of tumour cells and stimulates synthesis of MMPs 1, 2 and 3 by fibroblasts (Zucker et al., 2000). It may also bind MMP-1 to the surface of cancer cells (Guo et al., 2000). In animal models, EMMPRIN transfected breast cancer cells form larger tumours (with greater gelanolytic activity) and more metastases compared to their empty vector controls (Zucker et al., 2001). Nothing is known about EMMPRIN in pancreatic cancer.

6. Summary

Taken together this evidence demonstrates that cancer cells interact with tumour stroma at multiple levels. There are clues that some of these interactions are taking place in pancreatic cancer but there has been no definitive study addressing the central question regarding the regulation of myofibroblast growth, extracellular matrix synthesis and the effect this matrix has on cancer cell phenotype, in what is the quintessential desmoplastic tumour.

Part 8
Hypothesis and Aims

1. Aims

The broad objective of this research was to investigate the role of the desmoplastic reaction found in pancreatic cancer. There have been a variety of disparate studies (cited in the introduction), which give clues as to the role of pancreatic tumour stroma but none that satisfactorily answers the central question of why it is there and how it affects cancer cell phenotype. The stroma is very well described but little is known about its functional consequence to the cells it envelops. This is particularly true of collagen, the quality and quantity of which changes most profoundly in pancreatic carcinogenesis. Current evidence indicates that myofibroblasts (derived from pancreatic stellate cells) are the major source of collagen within the pancreas and the central aim of this thesis was to determine the nature and effect of interactions between pancreatic cancer cells, stellate cells and secreted collagen.

2. Hypothesis

Pancreatic cancer cells derive a growth and survival advantage through their interactions with pancreatic stellate cells and extracellular matrix that are found in the desmoplastic reaction

Specific hypothesis and aims are identified in the relevant chapters of experimental work.

Chapter 2

General Methods

A series of hypotheses were investigated using pancreatic cancer cell lines and primary cultures of PSC in a variety of tissue culture models. General methods are explained in this chapter and others in the relevant chapter. All standard laboratory chemicals and reagents were obtained from Sigma unless stated.

1. Pancreatic Cancer Cell lines

Three pancreatic cancer cell lines were purchased from American Type Culture Collection (ATCC) or European Collection of Cell Cultures (ECACC). They were guaranteed to be of original genotype and mycoplasma free. This was subsequently confirmed (see appendix 2).

a) MIA PaCa-2 is an undifferentiated cell line, with fibroblastic morphology, derived from a primary pancreatic tumour. It has a doubling time of 40 hours and a high degree of aneuploidy (Yunis et al., 1977; Liehr et al., 1990).
b) Panc-1 is also poorly differentiated with squamous epithelial morphology. It was derived from a primary tumour and has a doubling time of approximately 52 hours (Lieber et al., 1975; Liehr et al., 1990).
c) AsPC-1 was derived from ascitic metastasis (Chen et al., 1982). In vivo it produces tumours with variable glandular differentiation and abundant mucin (Chen et al., 1982).

They all have mutated p53, p16 and K-ras (Moore et al., 2001). Smad4 is reported to be mutated in AsPC-1 (Moore et al., 2001). All grow in adherent cultures.

2. Isolation and Culture of Pancreatic Stellate Cells

The process of stellate cell isolation and culture was essentially the same for rat and human cells.

Isolation of Human Stellate Cells

The process of acquiring human stellate cells was approved by the Southampton and Southwest Hants local research ethics committee (093/00). The protocol was based on a method originally described by Apte et al. and subsequently validated in the laboratories of Southampton University (Apte et al., 1998; Shek et al., 2002). Pancreas was obtained from patients undergoing pancreatic resection for pancreatic or peri-ampullary neoplasia or chronic pancreatitis. The operating surgeon provided 2-5g of normal pancreas distant from the resection margin and away from the tumour. The pancreas was morselated and digested in 20mls of

sterile Hank's Balanced Salt Solution (Lifetec) with calcium (HBSS+) containing 0.0009% w/v bacterial collagenase P (Cl. histolyticum, Roche) and 0.0015% w/v bacterial pronase (Strep. griseus, Roche) and gently agitated at 37°C for 45 minutes. The digested pancreas was then passed through a nylon membrane to remove larger debris. 0.0005% w/v DNAse (derived from bovine pancreas, Roche) was added to the filtrate before centrifugation at 514g for 7 minutes. The pellet of cells was resuspended in 2mls of 0.001% DNAse and added to 14.7% iodixanol (Optiprep, Axis Shield) v/v HBSS+. This suspension was then aliquoted into 15ml falcon tubes (Greiner) and plain HBSS+ layered on top, allowing density centrifugation (914g for 20 minutes at 4°C). The rotor was allowed to come to a natural halt, without brakes to avoid disruption of the density interface. By virtue of the relatively high lipid content and high membrane to cytoplasm ratio, stellate cells appeared as a fuzzy band between the Optiprep and plain HBSS+. These cells were harvested and thoroughly washed in HBSS+ prior to a further centrifugation at 514g for 7 minutes. The cell pellet was resuspended in 16% v/v Dulbecco's Modified Eagles Medium (DMEM (Autogen), with supplements listed in section 3) and cultured in cell culture flasks. 0.25-0.75x10^6 cells were routinely isolated using this technique.

Isolation of Rat Stellate Cells

PSC were also obtained from rats in order to pilot experimental protocols and avoid wasting human PSC. Male Sprague-Dawley rats were anaesthetised with phenobarbitone, by appropriately qualified staff (following Home Office guidelines) and laparotomised. The pancreas was removed and the animal exsanguinated by transection of the inferior vena cava or renal veins. The pancreas was immediately placed in HBSS+. The protocol described for isolation of human pancreatic stellate cells was then followed. The only difference in protocol was using lower concentrations of the bacterial collagenases (collagenase P 0.0006% v/v HBSS+/pronase 0.001% v/v HBSS+). 1-2x10^6 PSCs were obtained using this technique.

Confirmation of the phenotype of PSC

The isolated human PSC cells became adherent over 3 to 14 days. Initially the cells displayed vacuoles, typical of lipid storing quiescent PSC (Fig. 2.1A) (Apte et al., 1998; Bachem et al., 1998). Following adherence to the tissue culture plastic surface (TCP) they became activated. This was characterised by gradual loss of lipid droplets (Fig 2.1B) and development of cytoplasmic extensions that typically accompanies acquisition of myofibroblastic morphology (Fig. 2.1C). Following activation cells started to proliferate and when a sub-confluent monolayer had

developed, they were passaged (using trypsin as described below). Passaging removed potentially contaminating cells such as acinar cells or macrophages (Bachem et al., 1998). The myofibroblastic nature of the PSC was confirmed prior to using them in experiments by demonstrating strong αSMA expression by Western analysis (Fig 2.1D). The relatively low numbers of PSC isolated from human pancreas often necessitated their culture for at least 4 weeks before appropriate numbers were available for use in experiments, thereby relying on proliferation of the cell population. The phenotype of rat PSC has been documented in work previously published by the research group (Shek et al., 2002).

3. Tissue Culture

Cells were cultured according to established protocols (from either ATCC/ ECACC or research group). Cells were cultured in DMEM supplemented with 2mM L-glutamine (Lifetec), 0.375% sodium bicarbonate (Biomedia), 80U/ml penicillin, 80μg/ml streptomycin (both Lifetec) and 32μg/ml gentamycin (Hoechst). The media for MIA PaCa-2/Panc-1 cells was supplemented with 10% heat inactivated foetal calf serum (FCS, Lifetec) and PSC/AsPC-1 cells with 15% FCS ('normal' culture medium). Cells were cultured in humidified incubators (Triple Red) at 37°C with 5% CO_2 in standard tissue culture plastic (Greiner). Initially the cell lines were grown up and frozen in liquid nitrogen to provide a replaceable stock of cells. One million cells were suspended in 1ml FCS supplemented with 10% dimethyl sulphoxide (DMSO) and progressively frozen (20°C for 24 hours, -80 °C for 24 hours), then stored indefinitely in liquid nitrogen. When required the cells were rapidly thawed in a water bath at 37°C and the contents added directly to warm culture media.

Pancreatic cancer cell lines were kept in continuous culture for 2 months or 20 passages at which point they were discarded and a new batch thawed. The cells were always passaged once before use in experiments. PSC were used between passages 2-5. Most experiments were performed with the cells cultured in 0.5%FCS v/v DMEM supplemented to reduce the growth promoting effects of FCS.

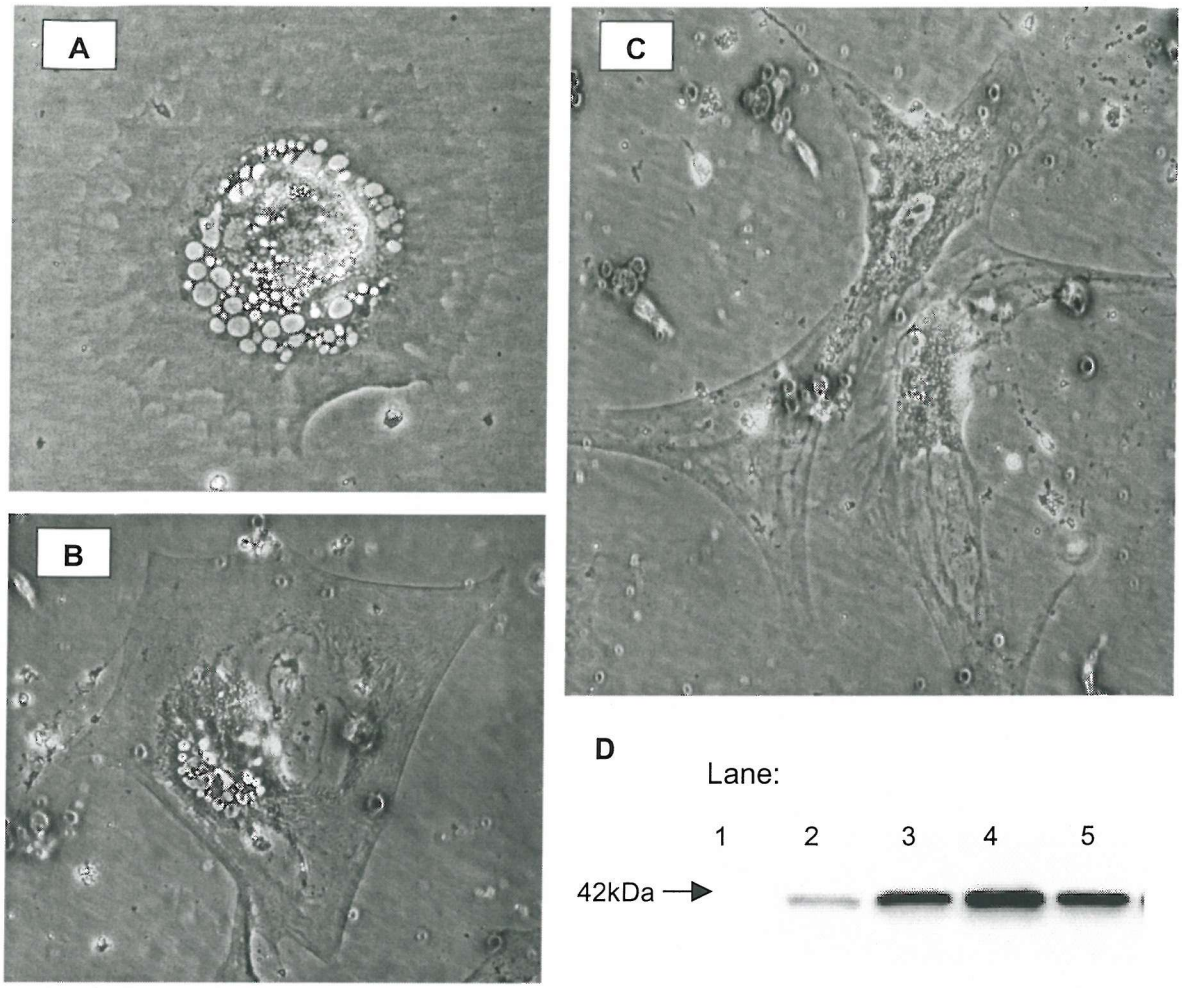


Figure 2.1
Confirmation of PSC phenotype
A. Photomicrograph of human PSC after culture on TCP for 4 days. There are numerous cytoplasmic lipid droplets contained in the cytoplasm (arrow), typical of quiescent phenotype. **B.** Photomicrograph of human PSC after 2 weeks in culture. The cells begin to lose the lipid droplets and develop cytoplasmic extensions. **C.** Photomicrograph of human PSC in culture after 3 weeks culture. Once the full myofibroblastic phenotype is evident, the lipid droplets have disappeared and there are pronounced cytoplasmic extensions. **D.** Western analysis of αSMA expression by cultured human PSC after 2-3 weeks of culture. Lane 1: MIA PaCa-2 (negative control), 2: HSC cell line (positive control), 3-5: 3 separate cultures of human PSC

4. Passaging Cells

PSC and cancer cells were passaged using standard techniques. Initially the culture medium was removed and the cells incubated in HBSS without calcium (HBSS-, Lifetec) for 10 minutes (this was repeated twice for stellate cells) at 37°C. The purpose of this was to remove any trace of FCS (which contains trypsin inhibitors) and disable calcium dependant intercellular adhesion molecules/receptors thus preventing cells detaching in sheets when trypsinised. Cells were incubated with 0.25% trypsin/EDTA (Biomedia) v/v HBSS- for

approximately 2 minutes, following which FCS was added and the cells centrifuged at 500g for 3-5 minutes. Cells were resuspended in culture medium using a Pasteur pipette and used in experiments or returned to culture.

5. Cell Viability and Counting

Prior to employing cells in experiments, their viability was routinely checked using the trypan blue exclusion technique. Following passage, an aliquot of cell suspension was stained with 0.0004% trypan blue (Sigma) v/v DMEM. Viability was routinely >95%. Cell numbers were determined by counting cells in a standard $1mm^3$ haemocytometer.

6. Experiments using purified extracellular matrix

All experiments using extracellular matrix were set up using an identical protocol.

Collagen

Purified collagens type I (from rat tail), type III (from calf skin) and type IV collagen (from human placenta, all Sigma) were made into 2mg/ml solutions by dissolving the collagen in 0.1M acetic acid (as per manufacturers instructions). Tissue culture plastic was coated with $15\mu g/cm^2$ of collagen (except where stated). Wells were coated by spreading the collagen solution over the plastic surface until it was evenly covered. The coated cell culture plates/flasks were then left at 4°C for 24 hours in order for the acetic acid to evaporate. Any residual acid was neutralised with DMEM. The protein structure of collagen is highly conserved amongst species and the technique of employing collagen (and other matrices) from different species is established laboratory practice (Boot-Handford and Tuckwell, 2003).

Matrigel

Matrigel is derived from a mouse sarcoma tumour model and contains type IV collagen and laminin (Wisdom, Jr. et al., 1992). It is stored frozen and has to be thawed slowly on ice or at 4°C. It requires quite careful handling because it readily forms a gel at room temperature and it needs to be maintained in liquid form to coat tissue culture plastic. Growth factor depleted Matrigel (Becton Dickinson) was diluted 1:2 with DMEM and TCP coated with $40\mu g/cm^2$ and incubated at 37°C for 1 hour, allowing gelation.

Laminin and Fibronectin

Laminin and fibronectin of human origin (both Sigma) were supplied diluted in PBS. Both were diluted with HBSS+ and used at $2\mu g/cm^2$ and $4\mu g/cm^2$ respectively (according to manufacturers instructions).

r/r Mutant Collagen

This was prepared from tails of r/r knockout mice according to methods of Cawson et al by Dr. X.Y. Zhou (Post Doctoral Research Fellow) (Cawston and Barrett, 1979). r/r Mice have a mutation in the α_1 chain rendering it resistant to collagenase cleavage and were a gift from Dr. S. Krane, Harvard Medical School, Boston, MA (Krane et al., 1996). Tissue culture plastic was coated with 15μg/cm^2 of r/r collagen as described above.

Tissue Culture Plastic (TCP)

TCP (all Greiner) was used as a neutral control in all experiments using extracellular matrix.

Optimisation of cell adhesion efficiency and specificity

TCP and extracellular matrices were routinely blocked with 1mg/ml bovine serum albumin for 1 hour (and washed off with HBSS-), prior to use in experiments. This was intended to stop non-specific binding. Manganese ions (Mn^{2+}) have been reported to increase constitutive activity of β_1 integrins, thereby enhancing integrin specific adhesion to extracellular matrix. Pilot experiments demonstrated Mn^{2+} also enhanced adhesion of pancreatic cancer cells to collagen (see appendix 3) and medium was therefore routinely supplemented with 0.1mM Mn^{2+} prior to seeding cells on different matrices (Bazzoni et al., 1995; Thamilselvan et al., 2003).

7. Measurement of DNA synthesis by ^{3}H-thymidine incorporation

DNA synthesis was assessed by tritiated thymidine (^{3}H-thymidine) incorporation and used as an indirect measure of cell proliferation (Boulton and Hodgson, 1995). Experiments were conducted in 24 well plates and each stimulus to proliferation was measured in triplicate. 1x10^4 pancreatic cancer cells or 1.5x10^4 PSC were seeded in wells of 24 well plates and incubated for 24 hours in normal medium. This was replaced with 0.5%FCS v/v DMEM eight hours prior to measurement of ^{3}H-thymidine incorporation, effectively depriving the cell cultures of external growth signals derived from FCS. The effect of supernatant transfer or cell culture on different extra-cellular matrices was then measured over 16 hours, following addition of 1μCi of ^{3}H thymidine (Amersham) to each well. Prior to scintillation counting, excess ^{3}H-thymidine was removed by washing the cells with cold HBSS+ (x3 for 10 minutes), with culture plates resting on ice. The cells were then fixed in methanol at –20°C for 30 minutes before being washed again with HBSS+ (x3 for 10 minutes). ^{3}H-thymidine was solublised in 0.25M NaOH/0.2M SDS, which was neutralised with equimolar HCl (to prevent chemiluminescence) and mixed with

scintillation fluid (Optiphase 'Hi Safe' 3 (Wallac). Scintillation was counted (2 minutes per well) with a Microbeta 1450 scintillation counter (Perkin Elmer). The mean counts/minute of each triplicate was used as the result for each condition tested. ^{3}H-thymidine incorporation depends on absolute cell numbers and to account for inter-experimental variation, scintillation counts were calculated as a percentage of the relevant control. Scintillation counts were only used if they were 10 times or greater the background counts (approximately 30 cpm). In practice the counts were rarely less than 1000 cpm. Coating TCP with extracellular matrix did not alter the background counts as a result of ^{3}H thymidine becoming adherent to the matrix.

8. Quantification of total DNA with PicoGreen reagent

PicoGreen ® dsDNA quantification reagent (Molecular Probes) is a fluorescent reagent that intercalates with double stranded DNA, allowing quantification of DNA, which can be used as an indirect measure of total cell number. This method was required to control for cell numbers used for making conditioned media or changes in proliferation and cell adhesion, whilst measuring different parameters (e.g. effect of cancer cell supernatant on MMP-2 synthesis by PSC). Cells were removed from culture by scraping the culture surface with a cell scraper, leaving cells suspended in the culture medium. This was transferred to an Eppendorf tube and the wells washed with phosphate buffered saline (PBS, Sigma), which was also added to the tubes (to ensure all cells were collected). Centrifugation (microcentrifuge 5000rpm) of the cell suspension generated a cell pellet, which was re-suspended in 100μL of 10mM Tris/1mM EDTA (pH 7.5)(TE buffer). Cell membranes were disrupted by sonification for 10 minutes in a water bath (Branson) and the samples transferred to a flat bottomed 96 well plate (Nunc). PicoGreen ® dsDNA quantification reagent 0.5% v/v TE buffer was added in equal quantities to the samples and fluorescence measured immediately using a CytoFluor ® microplate reader (wavelength of 480nm, excitation 485/20 and emission 530/30). In order to derive DNA concentration from fluorescence, a standard curve was generated from herring sperm DNA standards (Sigma) of known concentration, in each separate experiment. An example of a standard curve is shown below (Fig 2.2). In experiments requiring large numbers of cells, DNA was serially diluted to ensure that point would fall on the linear part of the curve. Coating the wells with extracellular matrix did not alter fluorescence.

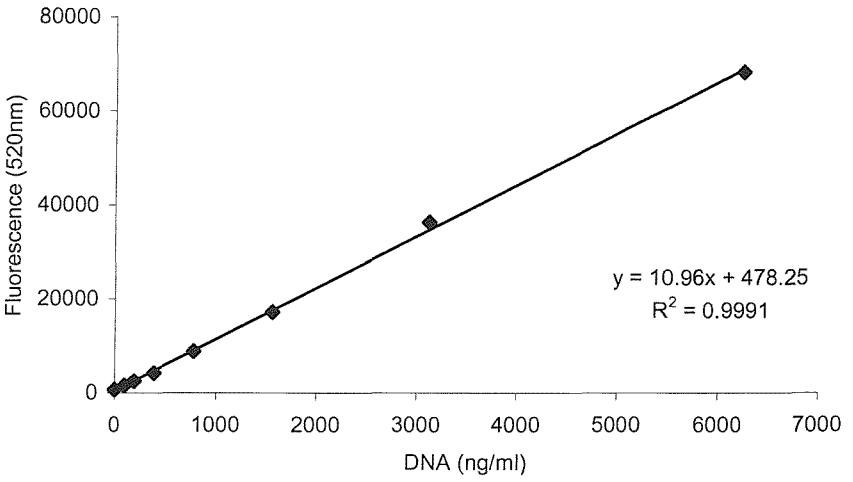


Figure 2.2
Example of standard curve generated from herring sperm DNA standards and measured with PicoGreen ® dsDNA quantification reagent
Standards were made by serial dilution of a known quantity of herring sperm in TE buffer. There was invariably good linear correlation (a trend line was generated using Microsoft Excel software, the formula of which was used to calculate DNA concentrations in unknown samples).

In ^{3}H-thymidine incorporation assays using extracellular matrix, DNA was measured from cells cultured in duplicate wells (on the same 24 well plate), to account for variations in adhesion efficiency amongst the cell lines. After removal of supernatant in the ^{3}H-proline collagen/MMP assays, DNA was measured in each cellular monolayer, to control for changes in cell number (following incubation with conditioned media). The effect this had on results is discussed where relevant.

9. Western Blotting

Western blotting was used to study protein expression in a number of tissue culture models. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used according to the protocol described below.

Protein preparation

Unless stated $8x10^{5}$ cells were seeded into 25cm^{2} flasks in normal medium and left for 24 hours, following which the cells were washed with HBSS+ and the remainder of the experiment conducted in 0.5%FCS v/v DMEM (with the exception of measuring αSMA expression by PSC). Cells were harvested by scraping the adherent cell monolayer (with a cell scraper) and collecting the culture medium. Cells were washed once in PBS and suspended in RIPA lysis buffer (0.15M NaCl, 5%NP40, 2.5% deoxycholic acid, 0.5%SDS, 0.25M Tris/HCL (pH 8.0), 1% mammalian protease inhibitors) in a volume approximately equal to the volume of

the cell pellet and then incubated on ice for 30mins. Cellular lysis was encouraged by intermittent vortexing. Debris (principally cell membranes) was cleared from the lysate by centrifugation (13,000rpm for 10 minutes). To ensure that equal quantities of protein were subjected to electrophoresis, the concentration of protein in the supernatant was measured using the Bicinchoninic Acid (BCA) Kit for Protein Determination (Sigma). This is based on the protein reduction of Copper (II) to Copper (I) in a concentration dependent manner. BCA is a specific chromogenic reagent for Copper (I) that permits colorimetric assay and consequently measurement of protein concentration in lysates. Standard curves were generated from standards made with known concentrations of BSA (see Fig. 2.3) from which the protein concentration was calculated. Following this step, protein was made to a final concentration of 2μg/ml with Red Loading Buffer (Molecular Probes)/ditiothreitol (DTT) and incubated at 95-100°C for 10 minutes, thus exposing epitopes by reduction of sulphide bonds.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Equal quantities (20μg with the exception of β-actin; 10μg and cyclin D_1; 60μg) of protein were resolved in pre-cast 4-16% gradient polyacrylamide gels (BioRad) by electrophoresis (200v) in electrophoresis buffer (25mM Tris, 192mM glycine, 3.5mM SDS), using standard Western blotting apparatus (BioRad). Molecular weight markers were routinely used (Cell Signalling).

Transfer of protein from gel to membrane

The gel was sandwiched between a sponge, blotting paper, nitrocellulose membrane (Schiener and Scheull) and then another sheet of blotting paper/sponge. The apparatus (BioRad) was then submerged in transfer buffer (25% absolute ethanol v/v electrophoresis buffer). The protein was transferred from the gel to 0.45μM nitrocellulose membrane over 1 hour.

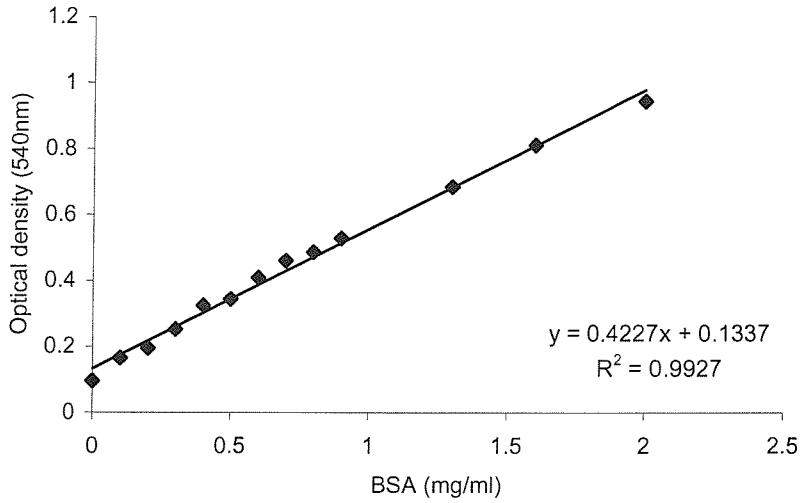


Figure 2.3
Example of standard curve generated from BSA standard measured using BCA protein assay kit
There was usually good linear correlation and a trend line was generated using Microsoft Excel software, the formula of which was used to calculate protein concentrations of the cellular lysates. The protein lysates were always serially diluted to ensure that optical densities did not exceed that of the standards.

Antigen detection and visualisation

All stages of antigen detection required reagents to be dissolved in either Tris buffered saline (0.4M Tris/HCl pH 7.4, 0.2M HCl) or PBS as indicated in table 2.1. Initially the membrane was blocked for 1 hour with 5% powdered low fat milk (Marvel) w/v buffered saline, then exposed to the primary antibody (raised against the target antigen, diluted in 5% milk w/v buffered saline) over night at 4°C. Membranes were washed 3 times in buffered saline prior to application of horse radish peroxidase (HRP) conjugated secondary antibody (species specific to the primary antibody, diluted 5% milk w/v buffered saline). After a further 2 hours membranes were washed a further 3 times in buffered saline. Buffers and antibodies used are listed in Table 2.1. Reactive bands were identified by applying a chemiluminescent substrate to the membrane (Super Signal, Pierce) and an image obtained using the Flour S Chemiluminesence Imaging System (BioRad). Occasionally images were obtained using autoradiography , where indicated (Kodak). [N.B. Western analysis of EMMPRIN required 10% milk w/v TS tween (TST, 0.05%Tween 20 (Sigma) v/v TS) for blocking and 1% milk w/v TST as antibody diluent].

Antigen	Buffer	Primary Antibody			Secondary Antibody	
		Antibody	Clone	Dilution	Antibody	Dilution
PARP	PBST	R&D Systems	C2-10	1 in 1000	Amersham sheep anti-mouse HRP conjugate	1 in 2000
EMMPRIN	TST	Santa Cruz	N-19	1 in 2000	Sigma mouse anti-goat HRP conjugate	1 in 20000
mcl-1	TS	Santa Cruz	S-19	1 in 400	Amersham donkey anti-rabbit HRP conjugate	1 in 5000
bcl-XL	TS	R&D anti-Bcl-X	AF800	1 in 1000	Amersham donkey anti-rabbit HRP conjugate	1 in 5000
PCNA	TST	Gift from Xiu Lu, Ludwig Institute for Cancer Research.	PC10	1 in 1000	Amersham sheep anti-mouse HRP conjugate	1 in 5000
p27	TST		SX5398	1 in 1000	Amersham sheep anti-mouse HRP conjugate	1 in 1000
p21	TST		SX118	1 in 1000	Amersham sheep anti-mouse HRP conjugate	1 in 1000
β-actin	TST	Sigma	AC-15	1 in 1000	Sigma goat anti-mouse HRP conjugate	1 in 5000
cyclin D1	TST	Santa Cruz	DSC-6	1 in 100	Amersham sheep anti-mouse HRP conjugate	1 in 500
αSMA	PBS	Sigma	1A4	1 in 1000	Santa Cruz goat anti-mouse HRP conjugate	1 in 1000
bax	TS	Santa Cruz	N20	1 in 400	Amersham donkey anti-rabbit HRP conjugate	1 in 500
bak	TS	Santa Cruz	N20	1 in 400	Amersham rabbit anti-goat HRP conjugate	1 in 2000
heat shock protein 70	TST	Santa Cruz	B6	1 in 1000	Amersham sheep anti-mouse HRP conjugate	1 in 1000

Table 2.1
Summary of antibodies used for Western Blotting
The primary antibodies were all monoclonal, with the exception of the anti-EMMPRIN antibody. PBST (0.05% Tween 20 v/v PBS).

10. Extraction and Preparation of RNA

Extraction of RNA from cells

RNA was extracted from cells cultured in by identical methods to those used for Western analysis. After washing the monolayer with PBS, cells were lysed *in situ* in 4mM guanidine isothiocyanate (GIT, Sigma). RNA was extracted from the lysate using the RNeasy® kit (Qiagen), using RNAse free glassware, plasticware and water. This method was found to be quick and reliable and resulted in RNA suspended in water.

RNA Quantification/Quality

An aliquot of the RNA solution was subjected to optical densitometry (Ultrospec 2100 Pro UV/Visible Spectrophotometer) to determine the RNA concentration. The purity of extracted RNA was assessed by the 260nm/280nm ratio (which measures the RNA/Protein ratio), which was considered satisfactory if >1.8. RNA is at the mercy of degradative RNAses and the integrity of the 18s and 28s ribosomal sub-units was regularly checked after extraction. RNA was mixed in equal volumes with nucleic acid electrophoresis sample buffer containing ethidium bromide (17% formaldehyde, 7% glycerol, 1% ethidium bromide all v/v MOPS+0.5%w/v bromophenol blue). RNA was then resolved in a 1% agarose gel made with MOPS buffer (Sigma) containing 5%v/v formaldehyde, at 80v in MOPS buffer. The gel was viewed using ultraviolet light and RNA was considered undegraded if the ribosomal bands were clearly visible (Fig 2.4).

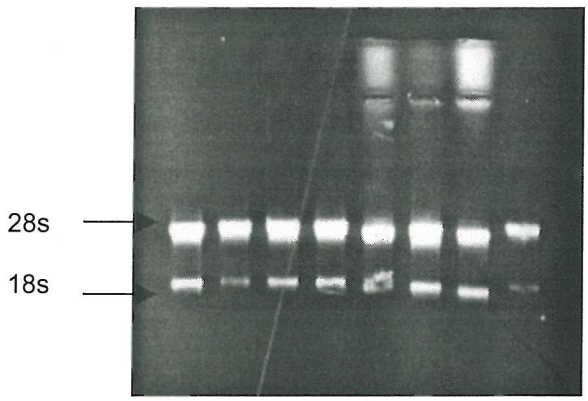


Figure 2.4
Example of RNA integrity gel
The integrity of RNA was checked prior to synthesis of first strand DNA. 18s and 28s denote the undegraded ribosomal subunits.

11. Synthesis of first strand cDNA

First strand cDNA was made from RNA using the Reverse Transcription System kit (Promega) exactly according to the manufacturers instructions. Initially the RNA was incubated at 70°C for 10 minutes to prevent any dimerised RNA entering the reverse transcription (RT) step. 1μg of RNA was added to a reaction buffer (comprising magnesium chloride, deoxy nucleoside triphosphates (dNTPs), ribonuclease inhibitors and random primers) and incubated at 42°C for 15 minutes. The reaction was stopped with incubation at 99°C for 5 minutes. If required optical densitometry was performed (as described for RNA) to determine DNA concentration.

12. Microscopy and Imaging

All microscopic images were obtained using Zeiss microscopes and processed using Zeiss Axiovision software. Images of zymograms were obtained using a Kodak digital camera and processed using Flour-S Imaging Software (BioRad). Other images were obtained using an office flatbed scanner and associated software (Hewlett Packard).

13. Biological Variation

Biological variation encountered between repetitions of an experiment was eliminated by normalising each result to that obtained with the control. Where this was necessary the results are presented as a percentage of control and is indicated in the accompanying text.

14. Statistical Analysis

Advice was sought from a university statistician (Dr. J. Goddard) and after a thorough explanation of the techniques it was felt that a 2 sample paired t-test would provide a robust statistical comparison between parameters tested in these experiments, despite necessitating assumptions of normal distribution of data. All analysis was done using Microsoft Excel Software.

Chapter 3

Histopathological examination of the desmoplastic reaction associated with ductal adenocarcinoma of the pancreas

Introduction

The histological changes associated with ductal adenocarcinoma of the pancreas have previously been characterised in a series of studies (Mollenhauer et al., 1987; Linder et al., 2001; Haglund et al., 1984; Yen et al., 2002). In the context of this study, however, it was important to study the manifestation of the desmoplastic reaction *in vivo* in order to inform and correctly interpret data from *in vitro* experiments. In view of previous anatomical studies of the desmoplastic reaction, this was limited to a small descriptive study, undertaken using immunohistochemical techniques. A significant desmoplastic reaction was present in nearly all ductal adenocarcinomas suggesting its development is a universal feature of the pathological process (Cruickshank, 1986). The extent of desmoplastic reaction has never been shown to correlate with prognosis however, but as all studies have been conducted on resection specimens (that comprise only 10-15% of all PDAC) this research is subject to systematic sample error. Interestingly the study of rectal cancers, which is not subject to the same degree of sample error by virtue of greater respectability rates, has shown the extent and nature of stroma adversely influences outcome (Ueno et al., 2002).

Hypothesis

The desmoplastic reaction alters the tissue architecture of the normal pancreas by increasing the collagen content and altering its composition, particularly with respect to fibrillar collagen.

Aims

1. To define the distribution of collagen type I, collagen type IV and overall collagen expression in ductal adenocarcinoma of the pancreas.
2. To identify the distribution and number of pancreatic stellate cells in the normal pancreas and compare this to ductal adenocarcinoma of the pancreas.

Methods

Appropriate approval from the Southampton and Southwest Hants local research ethics committee (138/02) was obtained prior to studying archival pathological

tissue. Patients who had undergone pancreaticoduodenectomy at Southampton General Hospital were identified from records and the formalin fixed and paraffin wax embedded tissue blocks were obtained. The actual staining of the pancreatic cancer sections was undertaken by the University Department of Immunohistochemistry, using established protocols.

1. Staining collagen with Sirius Red

Using an established protocol, 5μM thick paraffin embedded sections were dewaxed with xylene and rehydrated through graded alcohols until they were suspended in distilled water. After brief treatment with 0.2% phosphomolybdic acid, the sections were stained with 1% Sirius red w/v picric acid (both Sigma), washed, counterstained with Mayers haemalum (0.1% haematoxylin, 5% ammonium alum, 0.02% sodium iodate, 0.1% citric acid, 5% chloral hydrate v/v dH_2O (all Fisher)) , dehydrated and remounted.

2. Immunohistochemistry

Immunohistochemistry was undertaken using the same principles regardless of antibody used. The sections were deparaffinised with xylene and rehydrated through graded alcohol to 70%. Between each step of the process described below, the sections were washed twice with tris buffered saline (TS). Initially endogenous peroxidases were inhibited with 0.5% hydrogen peroxidase v/v absolute methanol.

Microwave proved to be the optimum pre-treatment for antigen retrieval for each of the antibodies used. This was undertaken by placing the dewaxed, peroxidase blocked sections in citrate buffer (0.1M made to pH 6.0 with NaOH) and incubated in a microwave oven for 25mins at medium power. Once cool the sections were washed 3 times in TS.

Sections were then blocked with a commercial Avidin-Biotin blocking kit (Vector Labs), followed by 20%FCS/1%BSA diluted in DMEM. The extra blocking step with FCS/BSA was always necessary when immunostaining the pancreas because of the wide range of antigens present. Sections were then exposed to the primary antibodies diluted in TS (Table 3.1) and incubated overnight at 4°C. Negative controls for this step included the use of isotype non-immune IgG (Sigma) or plain TS. Following this, species specific biotinylated second stage antibodies were applied (all Dako diluted 1:200 in TBS) for 30 minutes after which streptavidin biotin-peroxidase complexes (Menorini) were added. After further washing, the

sections were exposed to 3',3'-diaminobenzidine (Sigma) and finally counterstained with Mayers haematoxylin, dehydrated through graded alcohols and remounted.

Antigen	Retrieval Technique	Primary Antibody	Optimum Dilution
αSMA	microwave	Sigma clone 1A4	1 in 4000
Type I collagen	microwave	Abcam clone COL-1	1 in 100
Type IV collagen	microwave	Dako clone MO785	1 in 100

Table 3.1
Summary of antibodies employed in immunohistochemistry

Once stained the sections were analysed with Dr. Adrian Bateman (Consultant Histopathologist).

Results

Histological sections from six patients who had recently undergone pancreaticoduodenectomy for PDAC were selected. By definition the tumours were small, ranging from 2-4cm in diameter (bigger tumours are unresectable). The histological grade varied from well differentiated to poorly differentiated. All tumours displayed desmoplastic reaction but the degree to which this was present also varied. There was evidence of both peri-neural and vascular invasion amongst the tumours and lymph node metastases were present in 3 of the specimens. Normal pancreas was obtained from patients who had undergone pancreaticoduodenectomy for dysplastic duodenal polyps (familial adenomatous polyposis), duodenal cancer and an ampullary carcinoma arising from the common bile duct.

The quality of initial formalin fixation of the tissues was in some cases poor, due to the relatively large size of the paraffin embedded blocks and this was occasionally evident in some sections.

1. Epithelium

The cellular components of the pancreas were visualized by virtue of haematoxylin counter stain. The normal pancreas is comprised of numerous, regular acinar units

that comprise the bulk of the pancreas (best seen in Fig 3.1A and 3.1C). These are punctuated by Islets of Langerhans (Is, Fig. 3.1A). The larger duct cells are visible, particularly in longitudinal section (arrow Fig. 3.1D). The finely lobulated morphology of the pancreas is evident microscopically (Fig 3.2A).

This structure is grossly altered in malignancy. Large, occasionally very extensive, irregular glands with obvious lumina are seen (e.g. Fig. 3.2C). These demonstrate nuclear pleomorphism and occasional mitotic figures typical of malignant epithelium. There was a relative paucity of blood vessels in the stromal elements of the tumour.

2. Sirius Red stain for total collagen

Sirius red stains all types of collagen but particularly fibrillar collagens (types I and III). The pattern of collagen staining was profoundly different between the normal pancreata and pancreatic cancers (Fig. 3.2). In the normal pancreas the collagen was present in fine interlobular septa (arrow Fig 3.2A)). There were occasional delicate strands interposed between acinar units and surrounding Islets of Langerhans (Is, Fig 3.1A). There was more intense collagen staining around pancreatic ductules and the amount of collagen surrounding each duct increased with the size of duct (arrow Fig. 3.1A). Similarly collagen surrounds vascular structures within the pancreatic parenchyma.

The striking difference in the pancreatic cancers was the dramatic increase in collagen in the malignant tissue compared to that in the normal pancreas (Fig 3.2B-C). The predominantly cellular tissue of the normal pancreas is overrun with desmoplastic tissue. Wide tracts of collagen separate the malignant glands. Some bundles of collagen give the impression of organization, running parallel to each other, while in other areas it appears to be randomly distributed. There were numerous cells punctuating the areas of collagen, which are not present amongst the collagen seen in the interlobular septa of the normal pancreas. Many of these appear to be pancreatic stellate cells (see below) but would also include macrophages and lymphocytes (Emmrich et al., 1998). The interlobular septa in the surrounding pancreas become wider and there is a general increase in collagen staining, indicative of peri-tumoural pancreatitis. In some cases the intensity of the collagen staining reduces as the distance from the malignant focus increases.

3. Immunohistochemical staining for type I collagen

Type I collagen is a fibril forming interstitial collagen and it is an important structural component of most tissues. In the normal pancreas it was widely present within the periductular connective tissue, particularly the larger ducts (arrows Figs. 3.1D and 3.3A). It was also present within the fine fibrous septa separating lobules comprised of groups of acinar cells as well interdigitating between individual acinar units in some areas. Staining was present in the connective tissue supporting vascular structures and was weakly expressed within the walls of arteries and veins themselves (not shown).

In pancreatic cancer specimens, type I collagen was generally widely expressed within the desmoplastic stroma associated with infiltrating malignancy, showing a similar distribution to Sirius red staining (Figs 3.3B-3.3D). Its expression was most intense in areas of pronounced desmoplasia. Correspondingly it was less prominent in tumours where the epithelial component was less dominated by the desmoplastic reaction (e.g. Fig. 3.3C). There was no clear evidence of a gap being present between either the edge of either benign or malignant ducts and the collagen I fibers.

4. Immunohistochemical staining for type IV collagen

Type IV collagen is a meshwork forming collagen that normally gives structure to basement membranes. In the normal pancreas it was seen in delicate basement membranes surrounding single acinar units (Figs. 3.1C and 3.4A). It was also present to varying degrees in connective tissue surrounding ducts and blood vessels, as well as the interlobular septa. It was expressed at greater intensity in the blood vessel walls than type I collagen (not shown).

In the pancreatic cancer sections it was expressed widely in the desmoplastic stroma, associated with malignant glands (Figs 3.4B-D). The staining indicated somewhat finer strands amongst the bands of collagen, compared to the more diffuse staining observed with type I collagen. In some areas the basement membranes surrounding malignant ducts appeared to be discontinuous and irregular (arrows Fig 3.4B) but this was less pronounced in other cases (e.g. Fig 3.4C). Occasional basement membrane discontinuity was a feature noted around some ducts in sections of normal pancreas (arrow Fig. 3.4A). Furthermore, the type IV collagen staining around malignant glands was less defined than the acinar and ductular structures of the normal pancreas (Fig. 3.4).

5. Immunohistochemical staining for α-smooth muscle actin

In the normal pancreas occasional αSMA positive cells can be seen interspersed between acini, probably localised to interlobular septa. Furthermore the smooth muscle elements of the larger ducts and blood vessels stain intensely (arrows Figs 3.1B and 3.5A). It was not possible to exclude the possibility that there was staining of αSMA positive cells in close proximity to the larger pancreatic ducts. The mucosa muscularis of the duodenum stained positively for αSMA.

In contrast there is extensive αSMA staining throughout the desmoplastic reaction (Figs 3.5B-D). This varies in intensity. In some cases the staining was relatively sparse, localised to discrete spindle shaped cells (Fig 3.5D), whilst in others the staining was present more widely in the bands of fibrosis (Fig. 3.5B). In each case there was intense staining in the immediate vicinity of the malignant glandular tissue, with each gland appearing to be surrounded by αSMA positive cells (arrows Figs. 3.5B and D). Coupled to this there was relatively less αSMA staining in the central parts of the bands of desmoplasia (for example double-ended arrow Fig. 3.5C).

Discussion

These findings are consistent with previous studies, which have individually described the components of the desmoplastic reaction. The normal pancreatic architecture is grossly disrupted. The collagen content of pancreatic cancer is known to be much greater than that of the normal pancreas and this is clearly illustrated by staining the collagen in these sections with Sirius red. It has been shown previously that collagen type I and III comprise much of the desmoplastic extracellular matrix (Imamura et al., 1995a). We have demonstrated that type I collagen is widely present in comparison to the normal pancreas. Many of the malignant cells appear to be in contact with type I collagen, an observation supported by ultrastructural studies with electron microscopy, which demonstrate malignant cells in contact with fibrillar collagen, where basal lamina are discontinuous (Mollenhauer et al., 1987). In contrast, epithelial cells in the normal pancreas are generally in close contact to type IV collagen. Type IV collagen is also widespread in pancreatic cancer, which concurs with previous studies. Its distribution is rather diffuse and not confined to basement membranes. Previous research devoted to the study of basement membrane in pancreatic cancer consistently identified discontinuity (which correlated to tumour grade) compared to

continuous basement membranes in the normal pancreas (Mollenhauer et al., 1987; Haglund et al., 1984; Lee et al., 1994; Imamura et al., 1995b). We identified some discontinuity of staining around malignant glands but this was also a feature in some normal sections. It remains unclear whether epithelial or stromal cells are responsible for secreting basement membranes (Hagedorn et al., 2001). The discontinuity in malignancy may be a symptom of disordered epithelial metabolism, a breakdown in epithelial:mesenchymal interplay or abnormal degradation of basement membranes by proteases (or a combination).

If the relative distribution of type I and IV collagen in the normal and malignant pancreas is compared, it is immediately obvious that the increase in type I collagen is proportionally greater than type IV collagen. This pattern of fibrosis, characterised by accumulation of type I and other fibrillar collagens is consistent with stellate cell mediated fibrosis in both chronic pancreatitis and liver cirrhosis (Bachem et al., 1998; Iredale, 2001).

Recent reports have indicated a relative overgrowth of pancreatic stellate cells in pancreatic cancer and we confirmed these findings (Yen et al., 2002). It was interesting to detect αSMA staining around the ductal components of the normal specimens, although it could have been expected for 2 reasons. Firstly, larger ducts are surrounded by smooth muscle cells and myofibroblasts, which occupy a position in the periductular connective tissue, intimately related to the duct structures (Egerbacher and Bock, 1997). Secondly, the ducts are nourished by remarkably complex vascular plexuses, which may also contain smooth muscle (Egerbacher and Bock, 1997). Furthermore, mild obstruction of pancreatic ducts has been shown to induce periductular αSMA expression by PSC (Suda et al., 2000; Kishi et al., 2003). The length of time and degree to which duct obstruction was present correlated with degree of fibrosis and prevalence of αSMA expression (Suda et al., 2000; Kishi et al., 2003). A degree of ductal obstruction was likely amongst the controls, which were taken from patients with peri-ampullary neoplasia, which despite not originating from pancreatic tissue, were likely to cause some proximal obstruction. The finding of widespread αSMA expression in the sections of pancreatic adenocarcinomas was in keeping with previous findings (Yen et al., 2002; Bachem et al., 2000). Furthermore, the observation of intense staining in the areas immediately surrounding the malignant glands was also made (Yen et al., 2002).

Conclusions

In contrast to the normal pancreas, tissue architecture is grossly distorted in ductal adenocarcinoma of the pancreas. This is characterised by wide bands of fibrosis containing fibrillar collagen and numerous myofibroblastic stellate cells with simultaneous loss of the normal pattern of basement membranes.

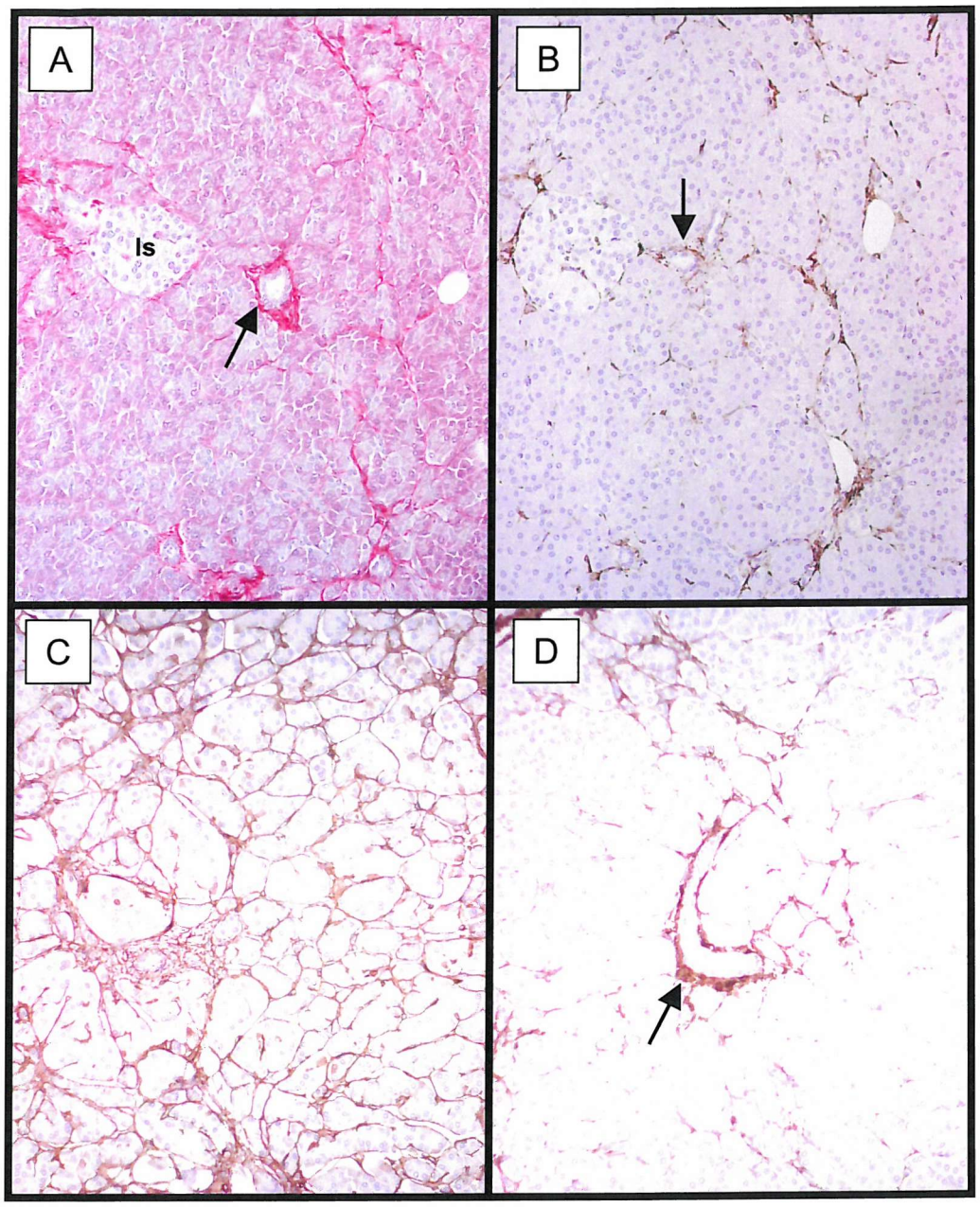


Figure 3.1
Normal pancreatic architecture
A. Normal pancreas stained with Sirius red (Is: Islet of Langerhans, arrow: pancreatic duct). **B.** Normal pancreas stained for αSMA (arrow: pancreatic duct). **C.** Normal pancreas stained for type IV collagen. **D.** Normal pancreas stained for type I collagen (arrow: pancreatic duct).

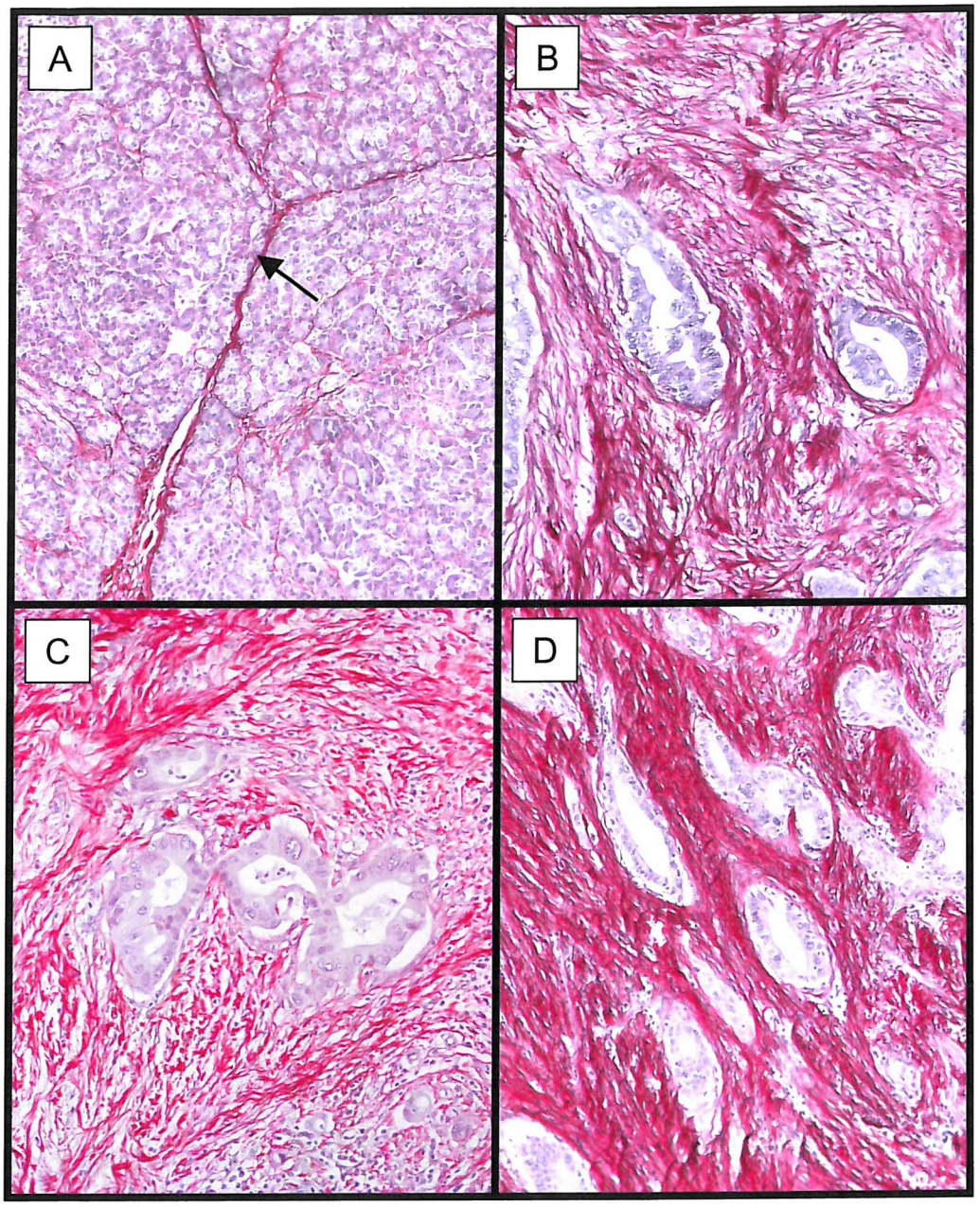


Figure 3.2

Normal pancreas and PDAC stained with Sirius red.

A. Normal pancreas (arrow: interlobular septum). **B-D.** Examples of PDAC.

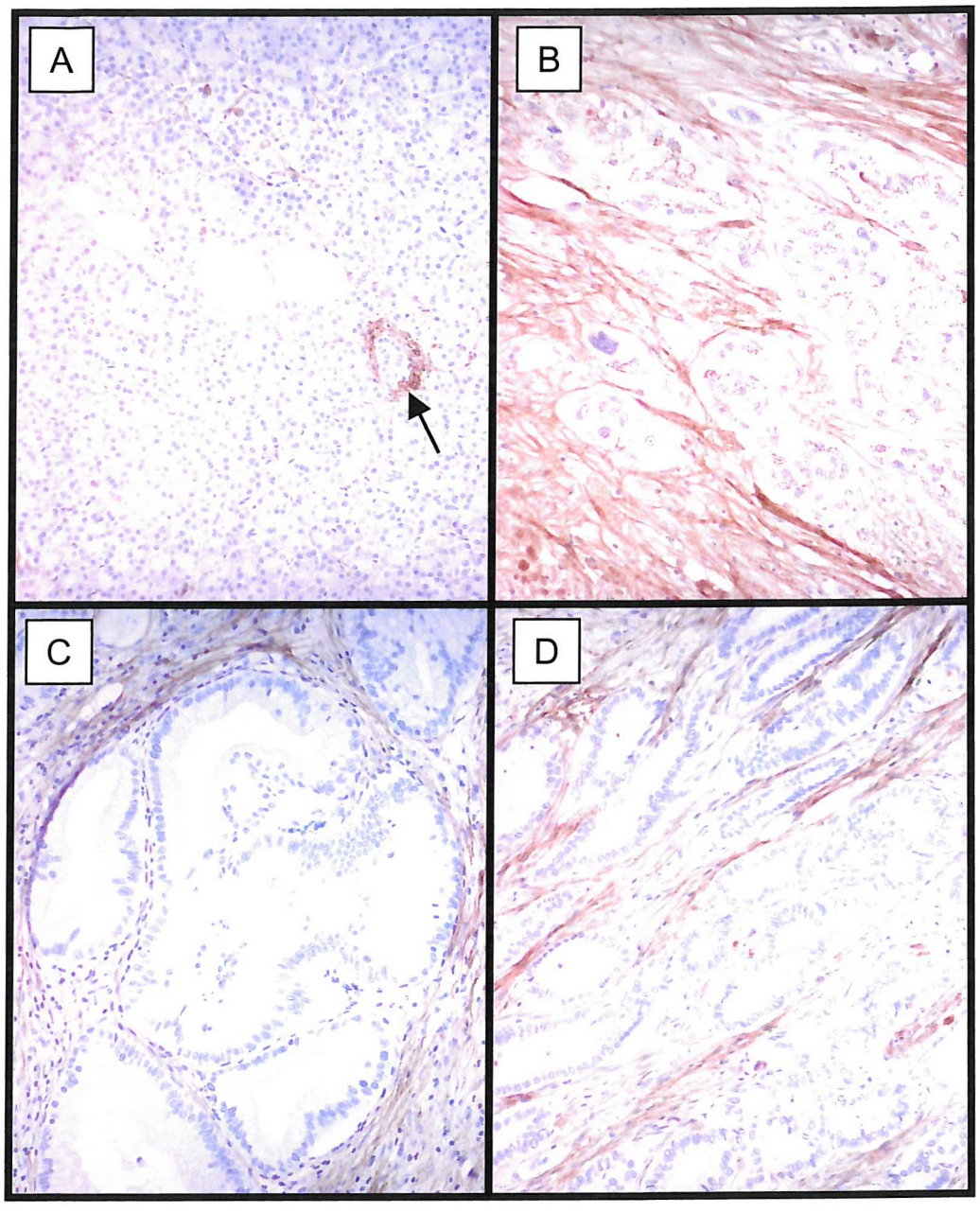


Figure 3.3

Normal pancreas and PDAC stained for type I collagen.

A. Normal pancreas (arrow: pancreatic duct). **B-D.** Examples of PDAC.

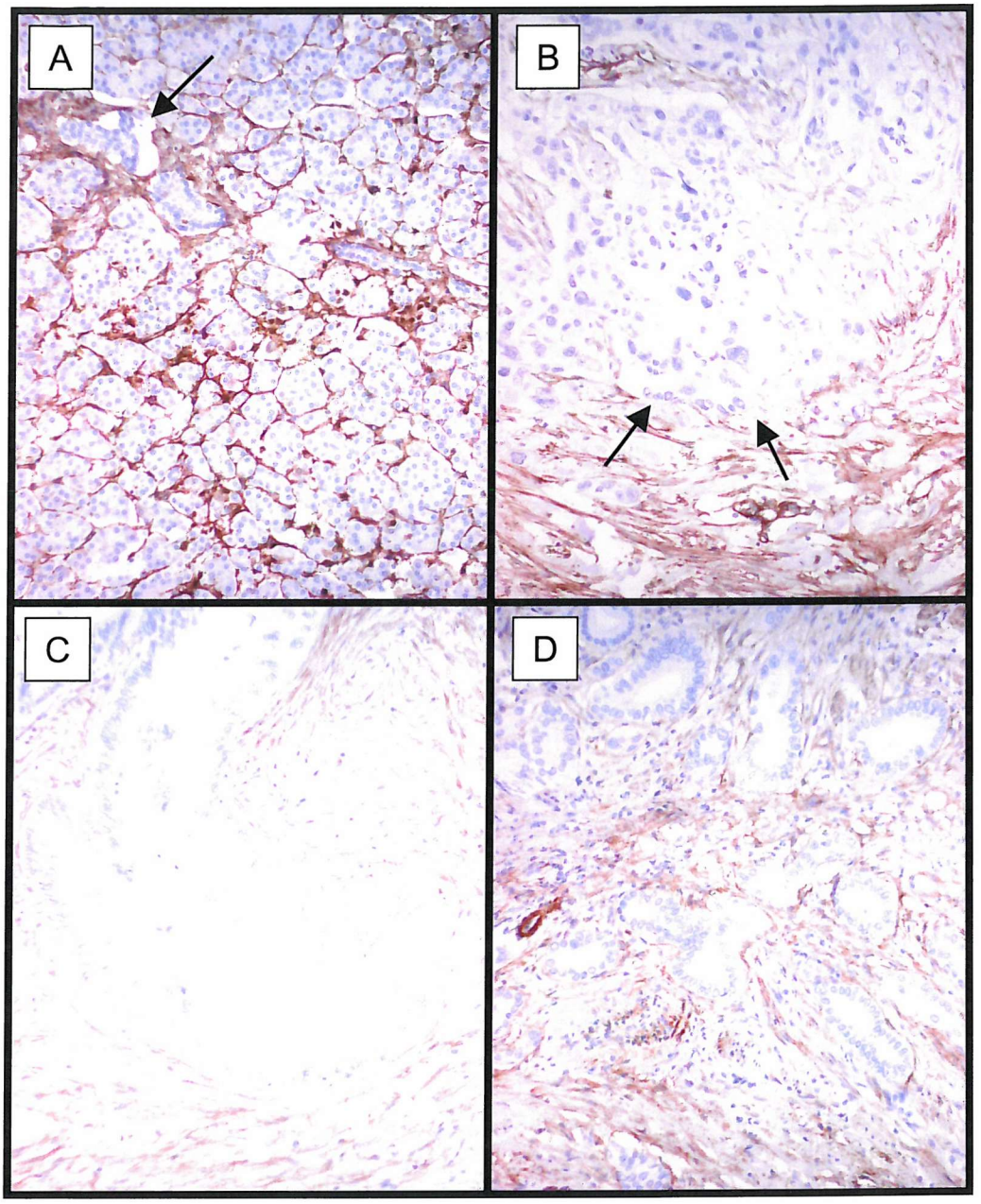


Figure 3.4

Normal pancreas and PDAC stained for type IV collagen.

A. Normal pancreas. **B-D.** Examples of PDAC. (arrows all indicate discontinuity in staining around a pancreatic duct in **A** and malignant gland in **B**).

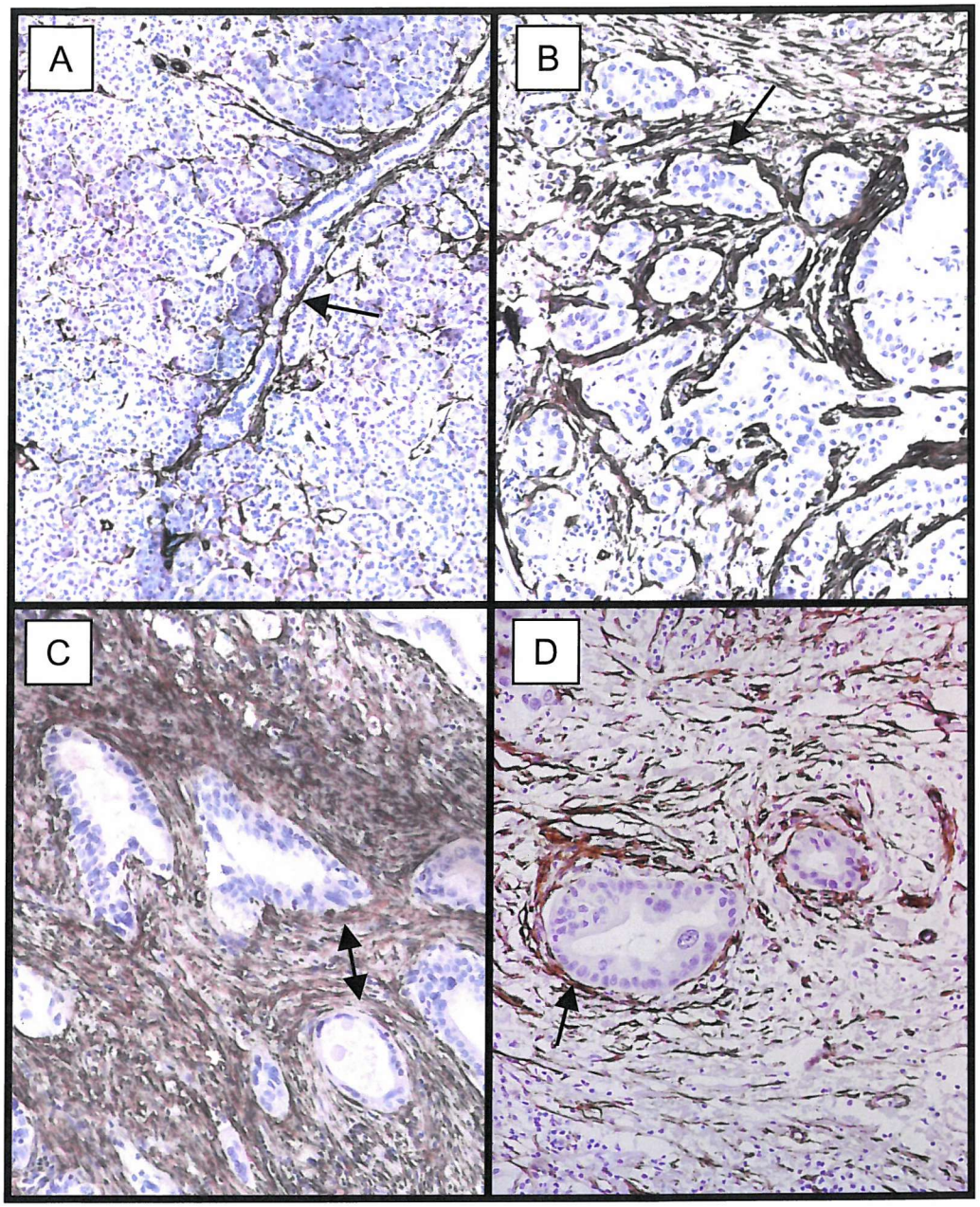


Figure 3.5

Normal pancreas and PDAC stained for αSMA.

A. Normal pancreas (arrow: pancreatic duct). **B-D.** Examples of PDAC. (arrows: **B** and **D** indicate intense staining around malignant glands and **C** the relatively weaker staining towards the center of thick bands of fibrosis).

Chapter 4

Investigation of functional interactions between pancreatic cancer cells and pancreatic stellate cells in the formation of the desmoplastic reaction

Introduction

The desmoplastic reaction, that characterises pancreatic cancer, grossly distorts normal tissue architecture, as demonstrated by histological studies presented in Chapter 3. The cellular players responsible for the origin of this phenotype and its development in pancreatic cancer remain ill defined. RNA transcripts for most extracellular matrix constituents have been detected in pancreatic cancer cell lines but at a lower level than fibroblast controls (Lohr et al., 1994). Furthermore, recent immunohistochemical studies have localised procollagen I to stellate cells and pilot data suggest that matrix secretion by stellate cells may be regulated by cancer cells (Gress et al., 1995; Yen et al., 2002; Bachem et al., 2000). Although MMPs and TIMPs have been identified in PDAC by in-situ hybridisation and immunohistochemistry, little is known about their cellular origin within the tumours or their potential roles in matrix turnover in the desmoplastic reaction.

Hypothesis

The interaction between pancreatic cancer cells and pancreatic stellate cells leads to the collagen rich desmoplastic reaction.

Aims

1. To determine if supernatant exchanged between pancreatic cancer cells and PSC alters the rate of proliferation of either cell type.
2. To investigate if cancer cells regulate stellate cell collagen synthesis.
3. To determine the relative expression of gelatinases (MMP-2 and 9) and TIMP-1 by pancreatic cancer cells and PSC.

Methods

Two tissue culture models were used to study the interaction of PSC and pancreatic cancer cells *in vitro*. The first was transfer of conditioned supernatant between cell type and the second was to directly co-culture PSC with cancer cells.

1. Tissue Culture Models

Conditioned medium

Conditioned medium was made from cancer cells and PSC using the same method. Cells were cultured in normal medium in a 75cm^2 flask until sub-confluent, following which the monolayer was washed twice with HBSS+. DMEM containing 0.5%FCS or 0.01%BSA was then conditioned for 24 hours. The medium was then removed and clarified, firstly by centrifugation and secondly by filter sterilisation (0.2 μM). Based on evidence from pilot experiments (see Fig. 4.3 later in Chapter 4) the conditioned medium was mixed in equal parts with fresh DMEM (containing 0.5% FCS or 0.01% BSA) to ensure fresh nutrients and maintain buffering capabilities, then stored at –80°C until required. The cells were kept and total DNA determined using PicoGreen reagent by the method described in Chapter 2, thus allowing a broad comparison of cell numbers conditioning the supernatant. The mean quantity of DNA derived from the monolayer of cells used to condition the supernatant did vary: Panc-1; 89.3μg, MIA PaCa-2; 105.6μg and AsPC-1; 135.1μg. This reflected the relative cell sizes and consequent occupation of surface area within the flasks (i.e. Panc-1 cells are largest and therefore the total number was fewer for a given surface area). As results were accumulated, using the supernatant, it became apparent that the supernatants had broadly the same effect and it was decided to accept their formulation on a volume basis rather than complicate matters by further correction for cell number (except in the MMP/TIMP-1 assay, see below).

Supernatant Transfer Model

The aim of this model was to simulate paracrine interactions that may occur *in vivo*. Cancer cell conditioned medium was transferred to PSC to determine its effect on PSC proliferation, collagen synthesis, gelatinase secretion and TIMP-1 expression. PSC conditioned medium was only studied in the context of cancer cell proliferation. There were not sufficient human PSC to routinely make conditioned medium from and therefore rat PSC conditioned medium was used as a surrogate. The same basic method underlay all assays involving supernatant transfer, specific differences are highlighted in table 4.1 and these should be referred to throughout the methods described in this chapter.

Experimental Variable		Assay			
		Thymidine Incorporation	Collagen Assays		MMP/TIMP-1 Assays
			Procollagen I TaqMan	Hydroxyproline Assay	
Culture Plate		24 well plate	25cm^2 flask	12 well plate	12 well plate
Initial Number of cells		1.5x10^4	6x10^5	2.5x10^4	2.5x10^4
Time in normal media		24hrs	until sub-confluent	until sub-confluent	24hrs
Type of conditioned media		0.5%FCS	0.5%FCS	0.01%BSA	0.5%FCS
Time in conditioned media prior to measurement of parameter		none	24 hours	24 hours	24 hours
Controls	**Negative**	0.5%FCS	0.5%FCS	0.01%BSA	0.5%FCS
	Positive	5%FCS	rTGFβ_1 (10ng/ml)	rTGFβ_1 (10ng/ml)	FCS/rMMPs
Method for controlling changes in proliferation		Not applicable	Equal quantities of RNA were used to synthesise 1st strand DNA	PicoGreen	PicoGreen

Table 4.1
Comparison of methods using transfer of conditioned media to study interactions between PSC and cancer cells
rTGFβ_1 (recombinant human TGFβ_1), rMMPs (recombinant MMPs).

Cells under investigation were seeded out in normal medium and incubated for at least 24 hours prior to removal of the medium thus allowing cells to adhere and begin growing normally. Cells were washed twice with HBSS+ (to remove traces of FCS) prior to treatment with conditioned medium.

Direct Co-culture Model

This model was employed to mimic cell:cell contact between PSC and cancer cells *in vivo*. In practice it was only reliably applied to the study of MMP and TIMP expression by the PSC and cancer cells. Initially 2.5x10^4 PSC were seeded into wells of 12 well plates (in normal medium). After 24 hours, 2.5x10^4 pancreatic cancer cells were added to duplicate wells containing PSC and left for a further 24 hours. At this point the cells were washed twice with HBSS+ and the medium replaced with 0.5%FCS v/v DMEM. For control purposes, PSC and cancer cells were cultured alone, for periods of time matching those used in co-culture. Supernatants from the cell cultures were harvested

after 24 hours incubation, centrifuged to clear debris and then studied for MMP expression. The cells remaining in the wells were numerated by measuring total DNA with PicoGreen (for control purposes).

2. Measurement of collagen synthesis by stellate cells

Collagen synthesis is regulated at two levels, firstly at a transcriptional level and secondly at a post-transcriptional level. This was reflected in the experiments performed. Recombinant human TGFβ_1 (Sigma) was used as a positive control in these experiments, as it is known to stimulate collagen synthesis in rat PSC (Shek et al., 2002).

TaqMan for pro-collagen I mRNA

The effect of cancer cell conditioned medium on PSC type I collagen mRNA expression was studied using TaqMan. Conditioned supernatant was applied to cultured PSC. After 24 hours RNA was extracted from the cells, its integrity confirmed and cDNA synthesised from equal quantities of RNA (as described in Chapter 2). Equal volumes of cDNA were then subjected to TaqMan Real Time- Polymerase Chain Reaction (RT-PCR), the principles of which are described in appendix 4. The TaqMan procedure was undertaken by Dr. L. Murphy (Post Doctoral Research Fellow), who had previously designed the procollagen I primers and probes and verified their amplification efficiencies and linearity. These were calculated by performing serial dilutions of template DNA and plotted against cycle thresholds (C_T) and the slope of the graph calculated, with efficiency $(E)=10^{[-1/slope]}$ (Pfaffl, 2001). The sequence of primers (used at 0.5μM) and probes (used at 1μM) employed in the TaqMan were for procollagen I: sense, 5'-caagaggaaggccaagtcgagg-3'; antisense, 5'-cgttgtcgcagacgcagat-3' (Oswel Laboratories) and probe, 5'-cctcaggtaccatgaccgagacgtgtggaaacc-3'. GAPDH sequences were: sense, 5'-gaaggtgaaggtcggagtc-3'; antisense, 5'-gaagatggtgatgggatttc-3' and probe, 5'-caagcttcccgttctcagcc-3' (Applied Biosystems). The reactions were conducted in Universal PCR Mastermix (Applied Biosystems) under the following conditions: initial hold at 50°C for 2 mins/95 °C for 10 minutes, then cycles of 95 °C for 15s (denaturing) and 60 °C for 60s (annealing). All reactions were performed in triplicate, and the results obtained for pro-collagen I, normalized to those obtained in parallel TaqMan quantification of mRNA for the housekeeping gene GAPDH (Pfaffl, 2001).

The equation used for this was:

$$R = \frac{E_{Target}^{[C_T \text{ Target (Control-Sample)}]}}{E_{Reference}^{[C_T \text{ Reference (Control-Sample)}]}}$$

Where R is result, Target is pro-collagen I, Reference is GAPDH, Control is untreated PSC and Sample is conditioned media/TGFβ_1 treated PSC.

Tritiated hydroxyproline assay

This method was based on that described by Agelli et al (Agelli and Wahl, 1988). PSC were treated with cancer cell conditioned media containing 0.01%BSA rather than 0.5%FCS (0.5%FCS led to technical failure probably due to blocking of the micropore plates (see below). The conditioned media/control media was supplemented with 25μg/ml ascorbic acid (Sigma) which stabilised newly synthesised collagen (Agelli and Wahl, 1988). After 24 hours 1μCi ^{3}H-proline was added to the cultures and left for a further 24 hours. Hydroxyproline is a very common amino acid in collagen and measuring ^{3}H-proline incorporation represents a sensitive way of measuring collagen synthesis at protein level but was not specific for any particular collagen type (Boot-Handford and Tuckwell, 2003; Agelli and Wahl, 1988). After 24 hours, the supernatant was removed and the cell layer kept for DNA measurement using PicoGreen analysis. The supernatant from each well was added to quadruplicate wells in a 96 well Micropore plate (Millipore). A solution of Tris/$CaCl_2$ containing N-ethylmalemide (a protease inhibitor), with or without bacterial collagenase (Cl. histolyticum, Worthington Biochemical Corporation) was added to 2 wells of the quadruplicate respectively. Newly synthesised collagen was degraded in wells containing collagenase, thus allowing any background scintillation to be measured (as tritiated proline may have been incorporated into other newly synthesised non-collagenous proteins). After this step, protein was precipitated using ice cold 50% trichloroacetic acid (TCA, Sigma) and collected on the micropore membrane at the bottom of the plate by means of a suction device. The precipitate was washed twice with 10% TCA and left to dry. Optiphase 'HiSafe' 3 cintillation fluid (Wallac) was added and scintillation measured using a Microbeta 1450 scintillation counter. An indirect measure of collagen synthesis per unit DNA in each culture condition was calculated using the following formula.

$$\text{Collagen} = \frac{\text{Mean Scintillation} - \text{Mean Background Scintillation}}{\text{Total DNA}}$$

This gave results in counts per minute (cpm)/ngDNA and the results for each condition were normalised to the untreated control, to account for interassay variation.

3. Measurement of TGFβ_1 in conditioned media

TGFβ_1 was measured in conditioned media from the cancer cells using a commercial TGFβ_1 Enzyme Linked Immunosorbent Assay (ELISA, R and D Systems), according to the manufacturers instructions. The conditioned medium (prepared as described above) was acidified with 1M HCl to release TGFβ_1 from its non-covalently bound latency associated peptide (LAP), thus producing the immunoreactive form. After neutralisation with 1.2M NaOH/0.5M HEPES aliquots were added to the ELISA plate, which was pre-coated with TGFβ receptor (type II). The ELISA relied on a standard sandwich format ending in colourimetric assay of a horseradish peroxidase substrate (tetramethyl benzidine (TMB)/hydrogen peroxide) after the reaction had been stopped by the addition of sulphuric acid. The measured optical density (at 450nm) was compared to a standard curve prepared with recombinant TGFβ_1 standards provided with the kit (Fig. 4.1).

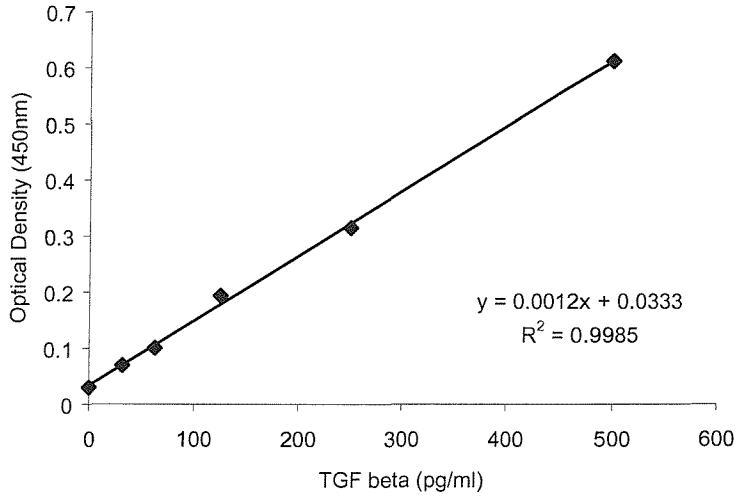


Figure 4.1
Standard curve generated from recombinant TGFβ_1

4. Measurement of MMP and TIMP expression

Gelatin Zymography

MMP-2 and MMP-9 expression was measured using gelatin zymography and commercial MMP activity assays. The ability of MMP-2 and MMP-9 to degrade gelatin underlies the principle of gelatin zymography. Initially 25μL of media from either supernatant transfer or co-culture model (see table 4.1) was mixed in equal parts with 2x zymography sample buffer (10% glycerol, 2% SDS, 13% 0.5M Tris/HCL pH 6.8, 0.013% bromophenol blue all v/v dH_2O) and subjected to SDS-PAGE on 8% polyacrylamide gels (0.01% SDS, 0.005%TEMED/ammonium persulphate v/v 0.125M Tris /HCL pH 8.8) containing 1% w/v gelatin, in a process identical to Western Blotting. Latent/ pro-MMPs are activated by SDS. The gels were removed from the electrophoresis apparatus, washed in dilute Triton X and dH_2O, then incubated in proteolysis buffer (50mM Tris/HCL pH 7.8, 50mM CaCl, 0.5M NaCl) at 37°C overnight. After rinsing the gels in water, they were stained with Coomassie blue (0.1% Coomassie blue, 10% acetic acid, 40% methanol v/v dH_2O). In order to identify areas of MMP proteolysis, the gels were then destained (7.5% acetic acid, 10%methanol v/v dH_2O) and images of the gels obtained using a digital camera (Kodak). Bands were compared to molecular weight markers (Cell Signalling). MMPs are calcium dependent and in order to ensure that observed proteolytic activity was MMP dependent, gels were also incubated in proteolysis buffer containing EDTA (50mM Tris/HCL pH 7.8, 10mM EDTA, 0.5M NaCl) prior to staining, to ensure specificity.

MMP Biotrack Activity Assays

In order to corroborate results from the gelatin zymography, MMP activity in the various conditioned media was measured in commercial kits according to the manufacturers instructions (MMP Biotrak Activity Assay, Amersham). The MMP activity assay relied on capture of latent and active MMP by anti-MMP antibody coated 96 well plates. Latent MMP was activated using p-aminophenylmercuric acetate (APMA). The captured MMP catalysed activation of a urokinase detection enzyme that degraded a colourimetric peptide substrate (S-2444™), the optical density of which is detected at 405nm. This allowed quantification of endogenously active MMP as well as total MMP by comparison to a standard curve generated from recombinant MMPs (Fig. 4.1). The MMP-2 kit worked well but the MMP-9 kit was not sensitive enough to detect the MMP-9 at the concentrations encountered in the conditioned media.

TIMP-1 Biotrak ELISA

TIMP-1 was also measured in the various supernatants using a commercial ELISA (Amersham), according to the manufacturers instructions. The conditioned media was diluted 1:100 prior to assay (based on data from pilot experiments).

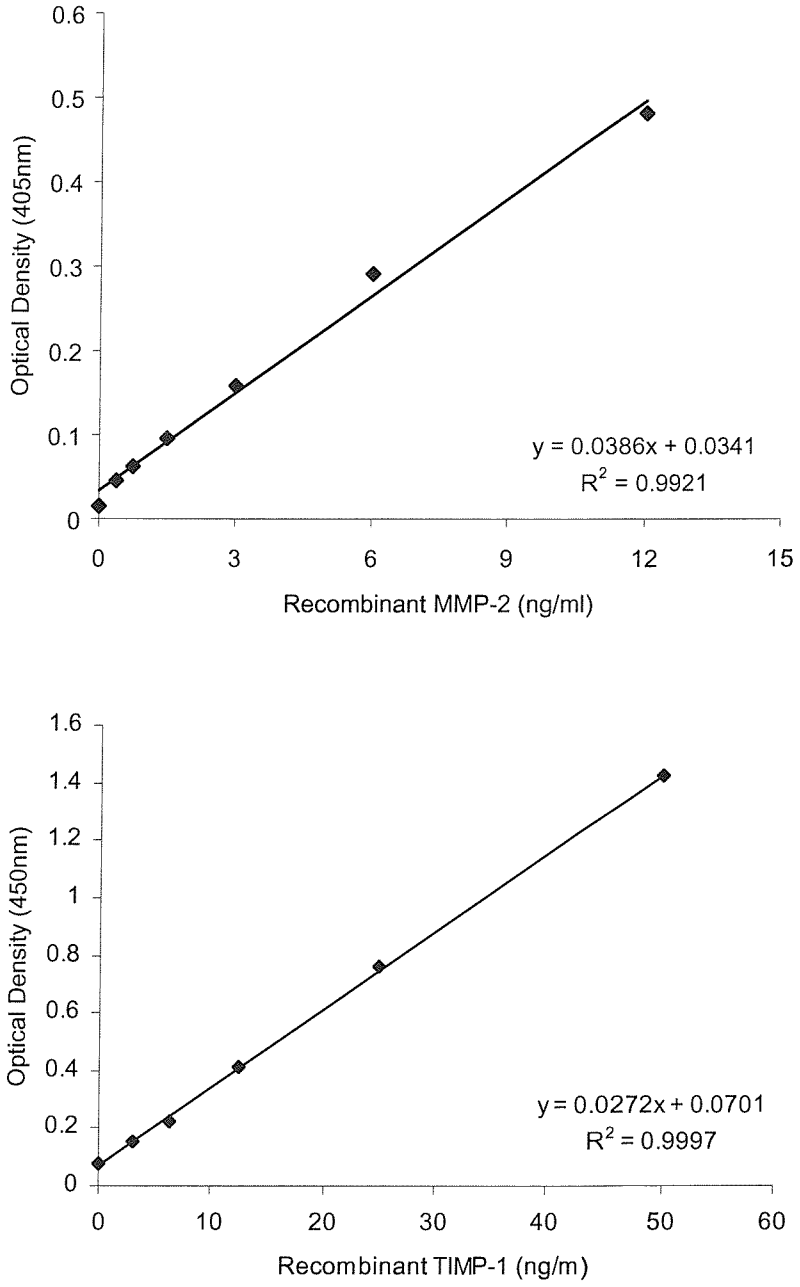


Figure 4.2
Standard curve generated from recombinant MMP-2 and TIMP-1
Supernatants were serially diluted to ensure that the measured optical density fell on the standard curve.

It was a simple sandwich ELISA format, which measured total TIMP-1 (including that complexed with MMPs). Captured TIMP-1 was identified by means of HRP conjugated anti-TIMP-1 antibodies that in turn degraded a colorimetric substrate (TMB/hydrogen

peroxide), the optical density of which was measured 450nm (after stopping the reaction with sulphuric acid). TIMP-1 was quantified in the conditioned media by comparison to a standard curve generated with recombinant TIMP-1 (Fig.4.2).

The results from both Biotrak assays were normalised to DNA (measured using PicoGreen) to account for changes in number of cells during the course of the assay. The equations used to derive comparable results from this information are shown below.

$$\text{Supernatant Transfer (pg/ml/ngDNA)} = \frac{\text{MMP-2(ng/ml)}}{\text{Total DNA from PSC (ng/ml)}}$$

$$\text{Supernatant Alone (pg/ml/ngDNA)} = \frac{\text{MMP-2(ng/ml)}}{\text{Total DNA from cancer cells used to make supernatant (ng/ml)}}$$

$$\text{Direct Co-culture (pg/ml/ngDNA)} = \frac{\text{MMP-2(ng/ml)}}{\text{Total DNA from PSC-DNA from cancer cells alone (ng/ml)}}$$

$$\text{Culture Alone (pg/ml/ngDNA)} = \frac{\text{MMP-2(ng/ml)}}{\text{Total DNA from PSC or cancer cells alone (ng/ml)}}$$

The equation used to derive results from the co-culture assay limits interpretation somewhat because it cannot account for the cellular origin of TIMP-1 in the conditioned medium. Data is considered within these confines.

Results

1. Effect of pancreatic cancer cell conditioned medium on proliferation of PSC

In order to determine if the close anatomical relationship occupied by malignant cells and PSC *in vivo* resulted in any functional interactions that may influence the formation of the desmoplastic reaction, a series of experiments were undertaken using pancreatic cancer cell lines and primary cultures of activated human and rat PSC *in vitro.* Firstly the effect of pancreatic cancer cell conditioned medium on proliferation of human PSC was studied in the supernatant transfer culture model. PSC were serum deprived for 8 hours (synchronising PSC in G_0 phase), then treated with conditioned medium for 16 hours, during which time ^{3}H-thymidine incorporation was measured using methods described in Chapter 2 (Fig 4.3).

The conditioned medium from each cancer cell line stimulated ^{3}H-thymidine incorporation by PSC in a concentration dependent manner, with the maximal response from conditioned media diluted 1:1 with fresh media (minimal dilution). This finding provided the basis for using conditioned media diluted 1:1 with fresh 0.5%FCS for all remaining experiments (i.e. 50%) to ensure any biological effects were maximised. The 50% conditioned media increased human PSC ^{3}H-thymidine incorporation 4.9-6.3 fold (Fig 4.4A). FCS, known to be rich in growth factors and cytokines, was used as a positive control (5%FCS v/v DMEM) and it also stimulated thymidine incorporation by PSC. Together, this evidence suggested that the cancer cells were stimulating growth of PSC by a paracrine mechanism (probably mediated by growth factors).

Conditioned media from human cancer cell lines also stimulated ^{3}H-thymidine incorporation by rat PSC (used to develop the methods employed in these experiments) although of lesser magnitude than observed in the human PSC (Fig 4.4B). This finding was important because experiments to determine whether PSC conditioned media regulated cancer cell growth relied on rat PSC conditioned medium. The potential of human cancer cell conditioned medium to stimulate PSC of a different species provided information with which to interpret the effect of rat PSC conditioned medium on human cancer cell growth.

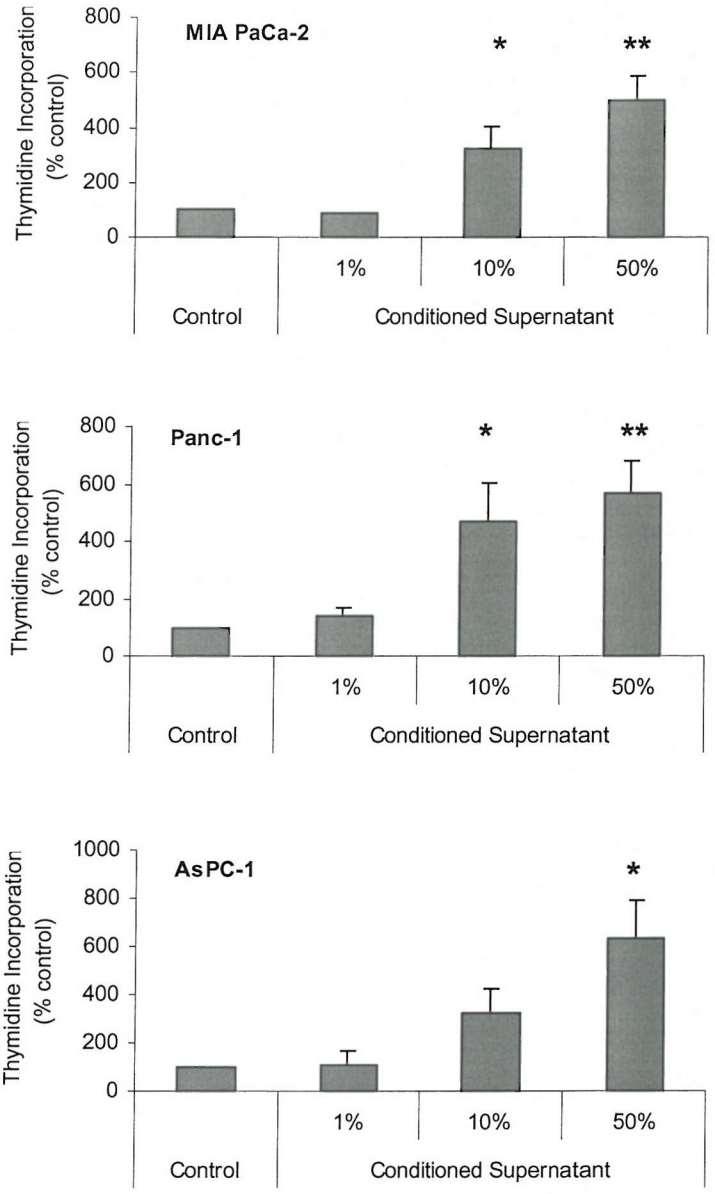


Figure 4.3
Effect of pancreatic cancer cell conditioned medium dilution on PSC ^{3}H-thymidine incorporation
Conditioned medium from MIA PaCa-2, Panc-1 and AsPC-1 cells was serially diluted with 0.5%FCS (used alone as a control) and transferred to human PSC. ^{3}H-thymidine incorporation was measured over 16 hours and it was increased in a concentration dependent manner by the conditioned media. The bars represent a mean of 6 independent experiments (n=2 for 1% concentration) +/-SEM, * $p<0.05$ and ** $p<0.01$, with respect to control.

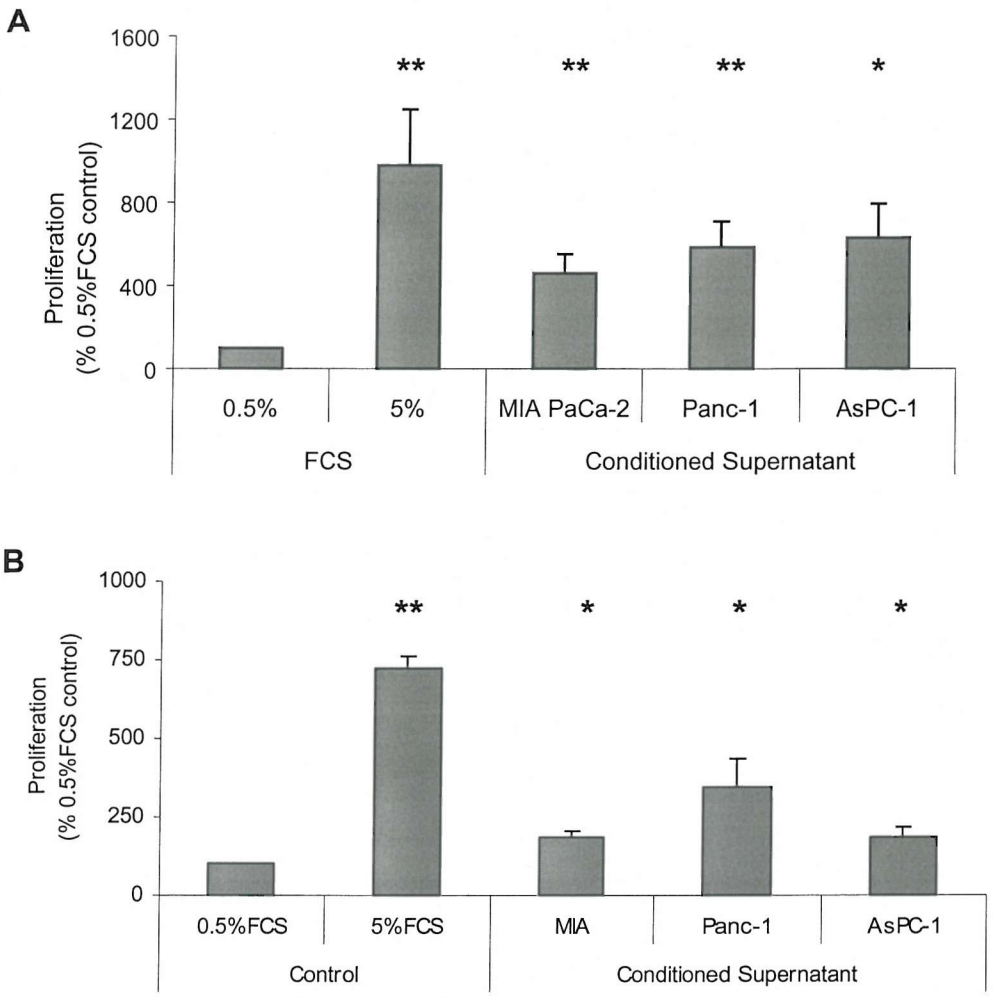


Figure 4.4
Effect of pancreatic cancer cell conditioned medium on PSC ^{3}H-thymidine incorporation
Conditioned medium (50%) from each pancreatic cancer cell line was transferred to **A.** human PSC and **B.** rat PSC and its effect on ^{3}H-thymidine incorporation compared to 0.5% and 5% FCS. The conditioned medium stimulated ^{3}H-thymidine incorporation compared to the negative controls but the magnitude was less for rat PSC compared to human PSC. The bars represent the mean of experiments on 6 separate isolates of human PSC (n=4 for rat PSC) +/-SEM, * $p<0.05$ and ** $p<0.01$, with respect to 0.5% FCS control. The mean absolute cpm obtained for the controls in this assay ranged from 402-999cpm in the human PSC and 1461-2411cpm for rat PSC.

2. Effect of PSC conditioned media on proliferation of pancreatic cancer cells

Human PSC were not isolated in sufficient quantities to permit conditioned media to be made. However, having established that cancer cell conditioned media had similar effects on ^{3}H-thymidine incorporation in rat PSC, conditioned medium from rat PSC was used as a surrogate to determine if PSC stimulated proliferation of the pancreatic cancer cells in the supernatant transfer model (Fig. 4.5). The conditioned medium modestly increased ^{3}H-thymidine incorporation in AsPC-1 cells but not the MIA PaCa-2 or Panc-1 cells. It is of interest to note the 5%FCS (used as a positive control) also

only stimulated ^{3}H-thymidine incorporation in AsPC-1 cells. This relative growth factor insensitivity may be due to either powerful autocrine growth stimulation or various genetic mutations permitting growth independence (discussed later).

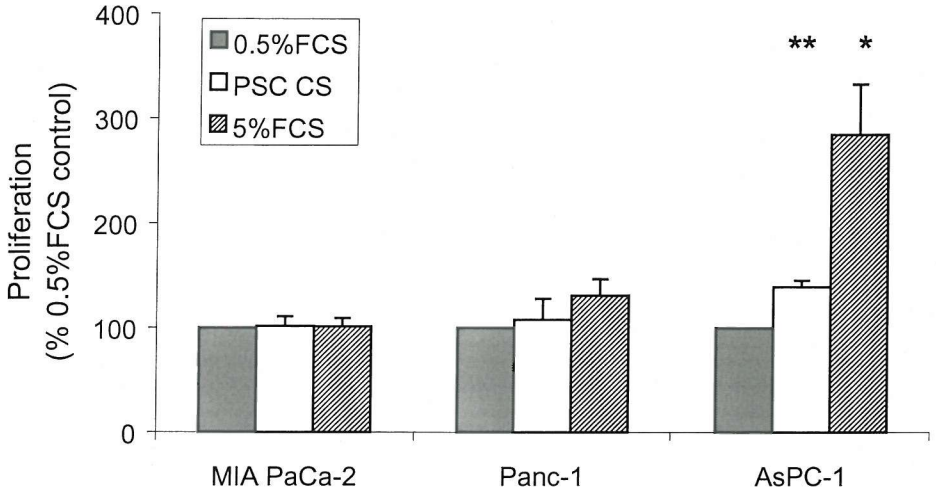


Figure 4.5
Effect of rat PSC conditioned medium on pancreatic cancer cell ^{3}H-thymidine incorporation
Conditioned medium from rat PSC (made from separate cultures of PSC diluted 1:1 with fresh 0.5%FCS) was transferred to the cancer cell lines and ^{3}H-thymidine incorporation measured. Rat PSC conditioned medium stimulated the growth of only AsPC-1 cells, over that of the negative controls (5% FCS was used as a positive control). In general the pancreatic cancer cell lines were relatively insensitive to FCS. The bars represent a mean of 3 independent experiments+/-SEM, * $p<0.05$ and ** $p<0.01$ with respect to 0.5%FCS control. The mean absolute cpm obtained for the controls in this assay ranged from MIA PaCa-2 301-1315cpm, Panc-1 538-837cpm and AsPC-1 402-862cpm.

3. Cellular origin of collagen in pancreatic cancer

There is plenty of circumstantial evidence to indicate that PSC are likely to be the source of collagen in the desmoplastic reaction of pancreatic cancer (Gress et al., 1995; Yen et al., 2002). However, transcripts for most matrix elements of the desmoplastic reaction have been demonstrated in pancreatic cancer cells (Lohr et al., 1994). The relative collagen secretion of the cancer cell lines and human PSC was therefore measured using the ^{3}H proline assay, described earlier (Fig. 4.6).

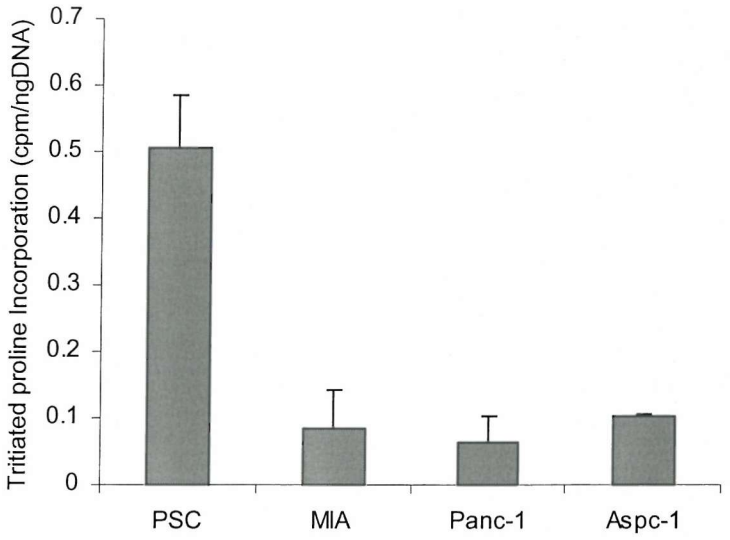


Figure 4.6
Relative collagen secretion of cultured PSC and pancreatic cancer cells
$2.5x10^4$ cells were seeded into wells of 12 well plates. After 24 hours the medium was replaced with 0.01%BSA v/v DMEM supplemented with ascorbic acid and ^{3}H-hydroxyproline incorporation measured over 24 hours. Results were corrected to DNA measured by PicoGreen reagent giving final results in cpm/ngDNA, then normalised to results obtained with PSC. Bars represent the mean of 2 separate experiments +/- SEM.

This assay indicated that PSC secreted collagen at 4.8-8.3 fold greater level than the pancreatic cancer cells.

4. Effect of pancreatic cancer cells on PSC collagen synthesis

The logical consequence of an increase in number of extracellular matrix secreting myofibroblasts would be increased matrix deposition. This would rely on maintenance of extracellular matrix secretion by PSC. To investigate if this was the case, the effect of cancer cell conditioned medium on human PSC procollagen I gene expression was studied using TaqMan RT PCR. Type I collagen was measured because its mRNA is reported to present at 3 fold the level of type III collagen mRNA in PSC and it dominates the desmoplastic reaction (Bachem et al., 2002). PSC were incubated with conditioned medium, plain 0.5%FCS or 10ng/ml TGFβ_1 v/v 0.5%FCS/DMEM for 24 hours prior to RNA extraction (TGFβ_1 is known to stimulate PSC collagen synthesis) (Shek et al., 2002). Equal quantities of this were used to synthesise first strand cDNA, which was subjected to TaqMan analysis (Fig. 4.7). The level at which procollagen I mRNA was expressed was normalised to expression levels of the house keeping gene GAPDH. As expected, procollagen I mRNA was readily detected in human PSCs, with a mean C_T of 20.7, compared to 26.0 for GAPDH.

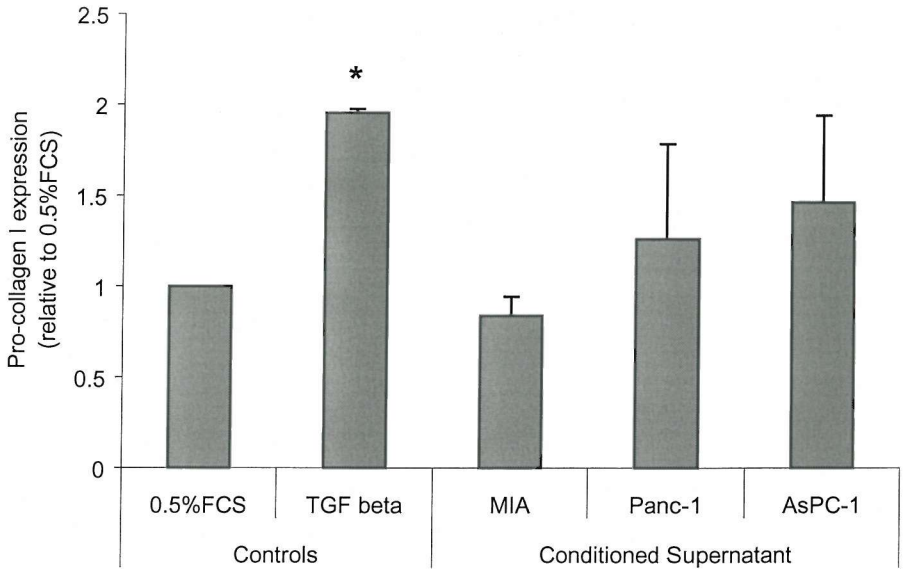


Figure 4.7
Effect of cancer cell conditioned medium on PSC procollagen I mRNA expression
Cancer cell conditioned media was transferred to human PSC and its effects on procollagen I mRNA expression measured by TaqMan RT-PCR. By measuring C_T and normalising it to GAPDH in parallel analysis (see methods), it was possible to compare procollagen I mRNA under each condition. The bars represent a mean of independent experiments, using 3 separate cultures of PSC. Recombinant TGFβ_1 10μg/ml w/v 0.5%FCS was used as a positive control (*$p<0.05$ with respect to 0.5%FCS control).

This indicated that recombinant TGFβ_1 significantly increased procollagen I mRNA in human PSC. Supernatant from Panc-1 and AsPC-1 cells also appeared to increase procollagen I expression but this assay suffered from some inter-experimental variability.

Collagen synthesis is known to be subject to extensive post-transcriptional control, its synthesis was therefore measured at protein level by studying PSC ^{3}H-hydroxyproline incorporation, again using the supernatant transfer model (Fig. 4.8) (Lindquist et al., 2000). The potential changes in PSC proliferation already identified in this tissue culture model were accounted for by normalising results to total DNA, quantified at the end of the assay using PicoGreen reagent (see general methods). The mean increase in DNA amongst the cultured PSC treated with conditioned medium was: MIA PaCa-2 7%, Panc-1 13%, AsPC-1 4% and TGFβ_1 9%.

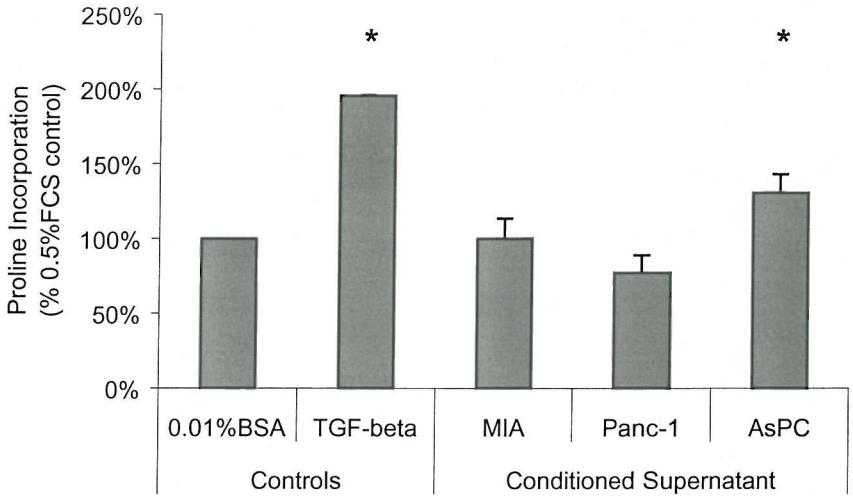


Figure 4.8
Effect of cancer cell conditioned medium on PSC collagen synthesis
Cancer cell conditioned medium was transferred to human PSC and ^{3}H-hydroxyproline incorporation measured over 24 hours. AsPC-1 conditioned media significantly increased collagen synthesis by human PSC but media from MIA PaCa-2 and Panc-1 cells had no effect. The data are presented as a percentage of control (0.01%BSA w/vDMEM) and given an arbitrary value of 100%, *$p<0.05$ with respect to control. Bars represent a mean of 3 or more independent experiments, recombinant TGFβ_1 10ng/ml was used as a positive control and also increased PSC collagen synthesis. The variation in absolute measurements amongst the controls ranged from 0.54-0.74cpm/ngDNA. It was not possible to use 0.5%FCS as a control in this assay because it appeared to block the micropore plates.

The conditioned medium from 2 of the cell lines increased procollagen I mRNA expression by human PSC and conditioned media from AsPC-1 cells led to a modest but significant increase in collagen synthesis at protein level.

In keeping with its known function, TGFβ_1 stimulated collagen secretion by PSCs. TGFβ_1 present in the cancer cell conditioned media was therefore measured using a commercial ELISA (Fig. 4.9). It was found that the relative ability of the cancer cell lines to stimulate either transcription of pro-collagen I at RNA level (Fig. 4.7) or total collagen synthesis (Fig. 4.8) correlated well with the concentration of TGFβ_1 present in the conditioned media.

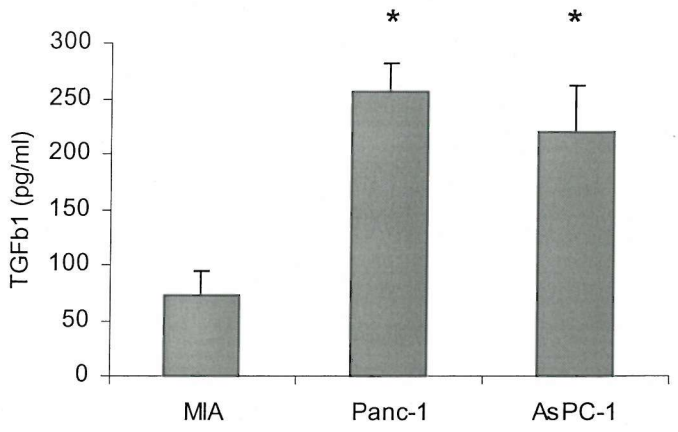


Fig. 4.9
TGBβ_1 concentration in conditioned supernatant from cancer cells
The total TGFβ_1 concentration in cancer cell conditioned media (used in experiments studying PSC collagen synthesis) was measured using an ELISA. Panc-1 and AsPC-1 cells secreted significantly greater amounts of TGFβ_1 than MIA PaCa-2 cells (*$p<0.05$). Bars represent the mean of 3 independent experiments +/-SEM. There was no TGFβ_1 measured in plain media (0.01%BSA v/v DMEM).

These results were corrected to DNA in order to confirm that TGFβ_1 was similarly expressed on a cell to cell basis. The mean TGFβ_1 for MIA PaCa-2 cells was 2.48+/-0.65 pg/ngDNA, Panc-1 9.66+/-1.08 pg/ngDNA and AsPC-1 8.68+/-1.94 pg/ngDNA, giving a very similar pattern to that in figure 4.9.

These data suggest that pancreatic cancer cells promote the expansion of the stromal compartment of PDAC by consistently increasing PSC number and therefore net extracellular matrix synthesis within the organ, while certain cell lines may have the potential to simultaneously increase PSC collagen synthesis by secreting TGFβ_1.

5. Expression and regulation of gelatinases and TIMP-1 in pancreatic cancer.
Extracellular matrix turnover is regulated by matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs). MMPs and TIMPs are widely expressed in pancreatic cancer and are implicated in oncogenesis (Chapter 1 Part 5.6). A novel role for stromal cells in tumours has recently emerged, in which they are regarded as an important source of MMPs in tumours and may inadvertently promote dissemination of cancer cells. The relative expression of MMPs and TIMPs by PSC and pancreatic cancer cells is unknown, however and it has clear implications in the formation of the desmoplastic reaction (as well as in the context of invasion and metastasis).

Expression of MMP-2 and 9 (gelatinases A and B respectively) and TIMP-1 expression was therefore studied in conditioned media from both a supernatant transfer model (employed above) and direct co-culture model *in vitro*. Potential variation in cell number was controlled for in both assays, by quantifying total DNA (using PicoGreen) and normalising results to it. A series of controls were undertaken for each individual assay and examples of these are shown below.

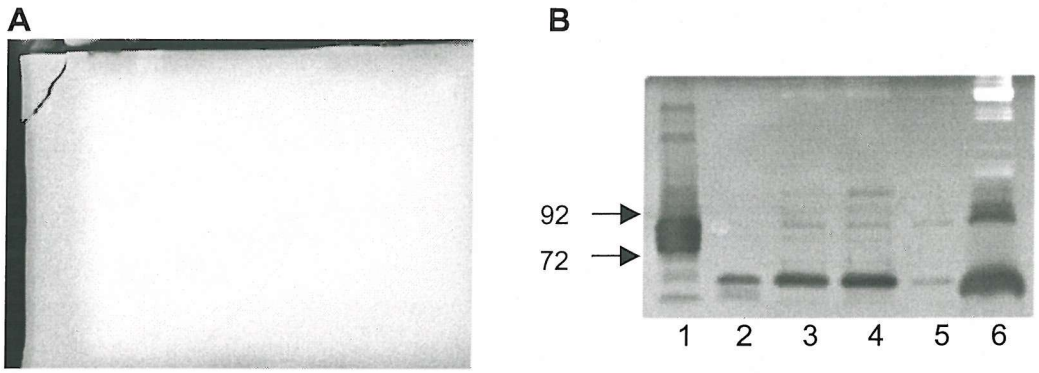


Figure 4.10
Controls used to interpret results of measured MMP expression
A. A duplicate zymography gel was made on 2 occasions and incubated with an EDTA rather than calcium containing buffer, to ensure gelanocytic activity was specific to MMPs, which this confirms. **B.** Controls for zymography: Lane 1) recombinant MMP-9, 2) recombinant MMP-2, 4) 0.5%FCS v/v DMEM, 6) FCS. Lanes 3 and 4 are supernatants from PSCs.

Total MMP-2 in 0.5%FCS v/v DMEM (in which all experiments were performed/ conditioned medium made) contained 0.1ng/ml MMP-2 (compared to a mean of 23.2ng/ml after conditioning with PSC) as judged by the Biotrak assay.

Initially MMP expression was studied using gelatin zymography (Fig 4.11). This demonstrated that human PSC expressed MMP-2 at greater levels than the cancer cells (with the exception of MMP-2 expression in Panc-1 supernatant (Fig. 4.11A) and to a lesser extent supernatant in the co-culture model (Fig. 4.11B) where equal cell numbers were used). Secondly MMP-9 was expressed at significantly lower levels by both cell types. There was no evidence that stellate cell MMP-2 or 9 expression was regulated by cancer cells in either the supernatant transfer or co-culture model. There appears to be an additive effect between PSC secreted MMP-2 and MMP-2 contained in Panc-1 supernatant (3rd lane Fig. 4.11A).

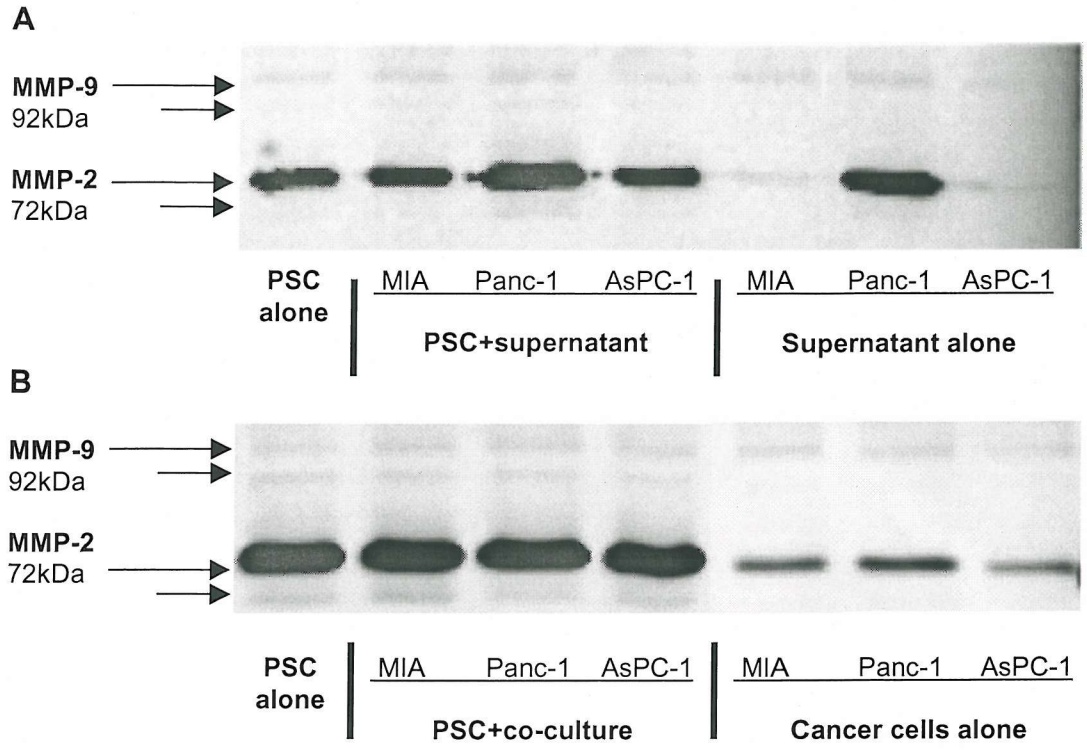


Figure 4.11
Expression of gelatinases by PSC and cancer cells determined by gelatin zymography
Supernatants were collected from PSC and pancreatic cancer cells cultured in supernatant transfer and co-culture models and 20μL separated on SDS-PAGE gels containing 1% w/v gelatin. The gelatinolytic bands obtained represent MMP-9 at 92kDa and MMP-2 at 72kDa. Activated gelatinases are indicated by the short arrows, weighing ~10kDa less after cleavage of the pro-peptide domain during activation. **A.** Demonstrates the MMP expression of human PSCs alone and those cultured with conditioned media from the cancer cells (as indicated). MMPs present in the cancer cell supernatant were also quantified (on the right). **B.** Demonstrates gelatinase expression in the co-culture model, with PSC alone on the left, co-cultured with each of the cancer cell lines, with relative MMP expression of the cancer cells alone on the right. Digital images were inverted using image analysis software (for presentation purposes), each is representative of experiments performed on 3 separate isolates of human PSC.

Gelatinase expression in supernatants from the same models was also measured using proprietary Biotrak ELISA based activity assays. The raw measurements for MMP-2 in the supernatants is shown below (Fig 4.12)

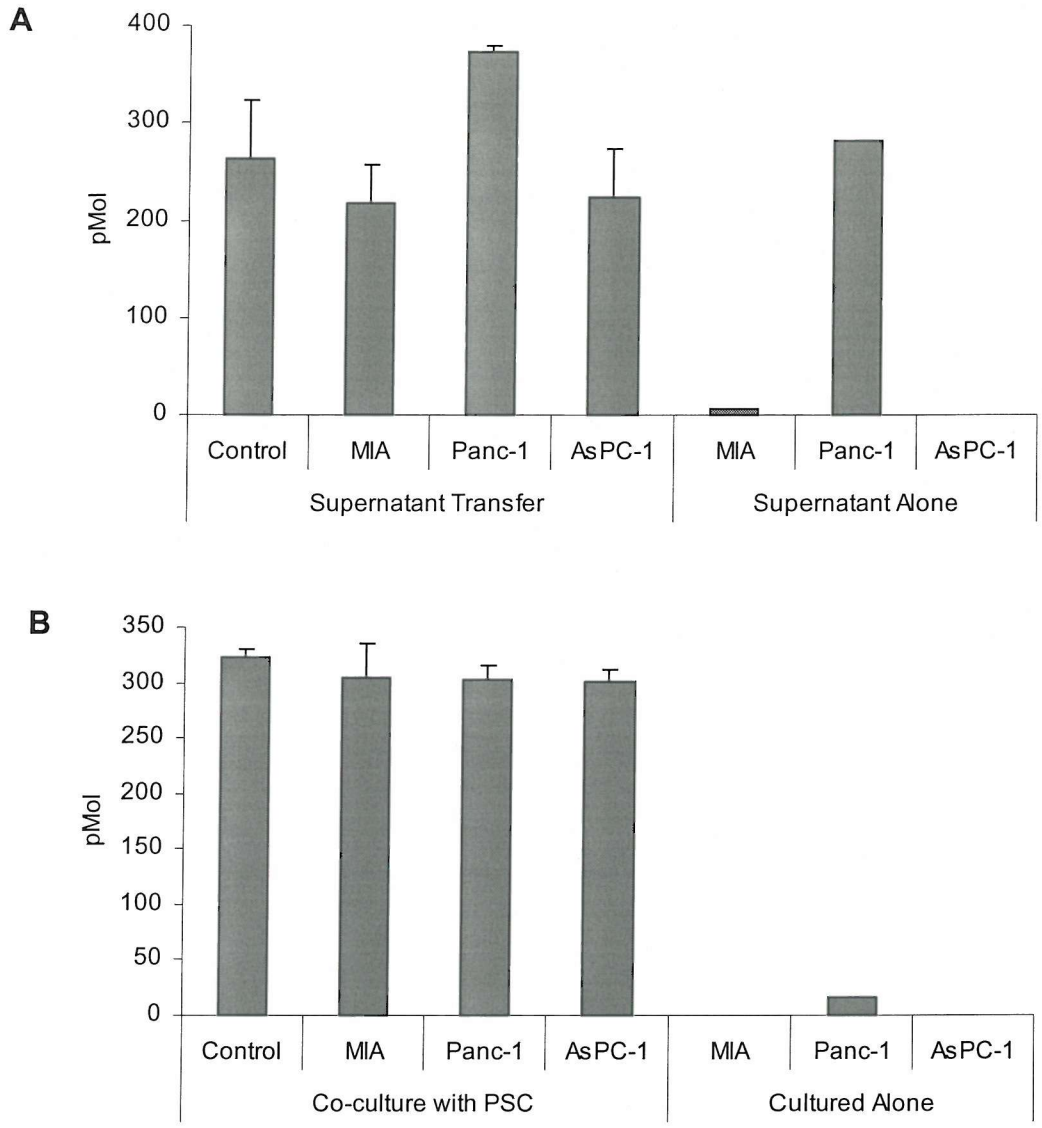


Figure 4.12
PSC MMP-2 expression in supernatant transfer and co-culture models
Pro-MMP-2 expression, measured using MMP-2 activity assay, in supernatants from **A.** the supernatant transfer or **B.** co-culture models, presented graphically in the same sequence as the zymograms. MMP-2 expression in supernatants of cancer cells and PSC cultured alone ('control') are also shown. Bars represent the mean of 3 independent experiments +/-SEM. All experiments were conducted in 0.5%FCS, which contained <0.01ng/ml MMP-2.

The results obtained using this assay accurately reflected the pattern observed in the zymograms, where the data was not corrected for cell number, showing consistency between the techniques. Furthermore, the Biotrak assay had the advantage of allowing the results to be easily normalised to total DNA, thus allowing the variation in proliferation of the PSC to be accounted for. It was also possible to measure endogenously active and total MMP by parallel analysis of native supernatant and supernatant activated with APMA respectively (see methods Chapter 4). The results

normalised to total DNA with endogenously active fraction of MMP-2 demonstrated, are shown below (Fig 4.13).

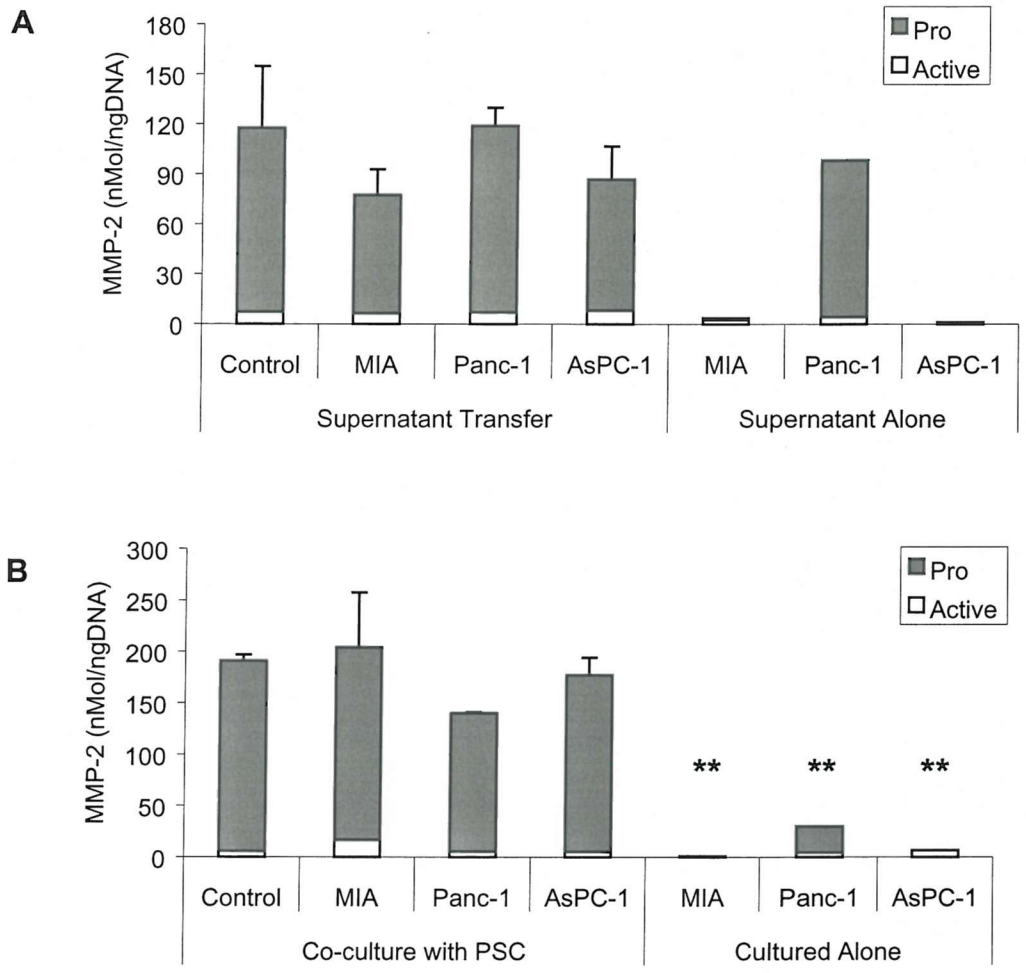


Figure 4.13
PSC MMP-2 expression in supernatant transfer and co-culture models normalised to DNA
MMP-2 expression in supernatants from **A.** the supernatant transfer or **B.** co-culture models, measured using MMP-2 Biotrak activity assay and normalised to total DNA (see equations in methods Chapter 4). MMP-2 expression in supernatants of cancer cells and PSC cultured alone ('control') are also shown. Bars represent the mean of 3 independent experiments (except MMP measured in the cancer cell supernatant (A), where n=1), with ** $p<0.01$ with respect to PSC controls. All experiments were conducted in 0.5%FCS, which contained negligible quantities of MMP-2. Error bars are SEM for total MMP-2 measured (those for the activated fraction were too small to demonstrate graphically).

Correction of results to total DNA was achieved using the formulae shown in the methods. It demonstrates that there was no consistent evidence of MMP-2 regulation that had been masked by changes in proliferation. There was some variation in the results obtained from co-culture and supernatant transfer to PSC, which reflects the complex nature of these assays.

The consistent finding in both tissue culture models and both assays was the marked differential in MMP-2 expression between PSC and the cancer cell lines. Panc-1 cells expressed MMP-2 and this was particularly evident in the conditioned medium used in supernatant transfer. There was no evidence of consistent regulation of PSC MMP-2 expression by either supernatant from or co-culture with any of the cell lines. The proportion of active MMP-2 secreted by the PSC was low (range 1.5-6% total) and was not altered by the cancer cells.

MMP-9 was measured using an activity assay but it was barely detectable preventing any meaningful results being obtained. This provided testament to the sensitivity of gelatin zymography, where MMP-9 in its pro and active forms was expressed at low levels by PSC and cancer cells alike. There was no evidence that cancer cells altered MMP-9 expression by PSC. It should be noted that 0.5% FCS v/v DMEM contained MMP-9 (see controls: figure 4.10) and it is not possible to be certain that MMP-9 was secreted over and above that by the cancer cells or PSC.

All secreted MMPs are subject to inhibition by TIMP-1 and it was therefore important to quantify TIMP-1 expression in the conditioned media. This was done using a commercial ELISA that detected total TIMP-1, including that bound to MMPs (Fig. 4.14). The results demonstrated that TIMP-1 was expressed at high levels by PSC compared to the cancer cell lines.

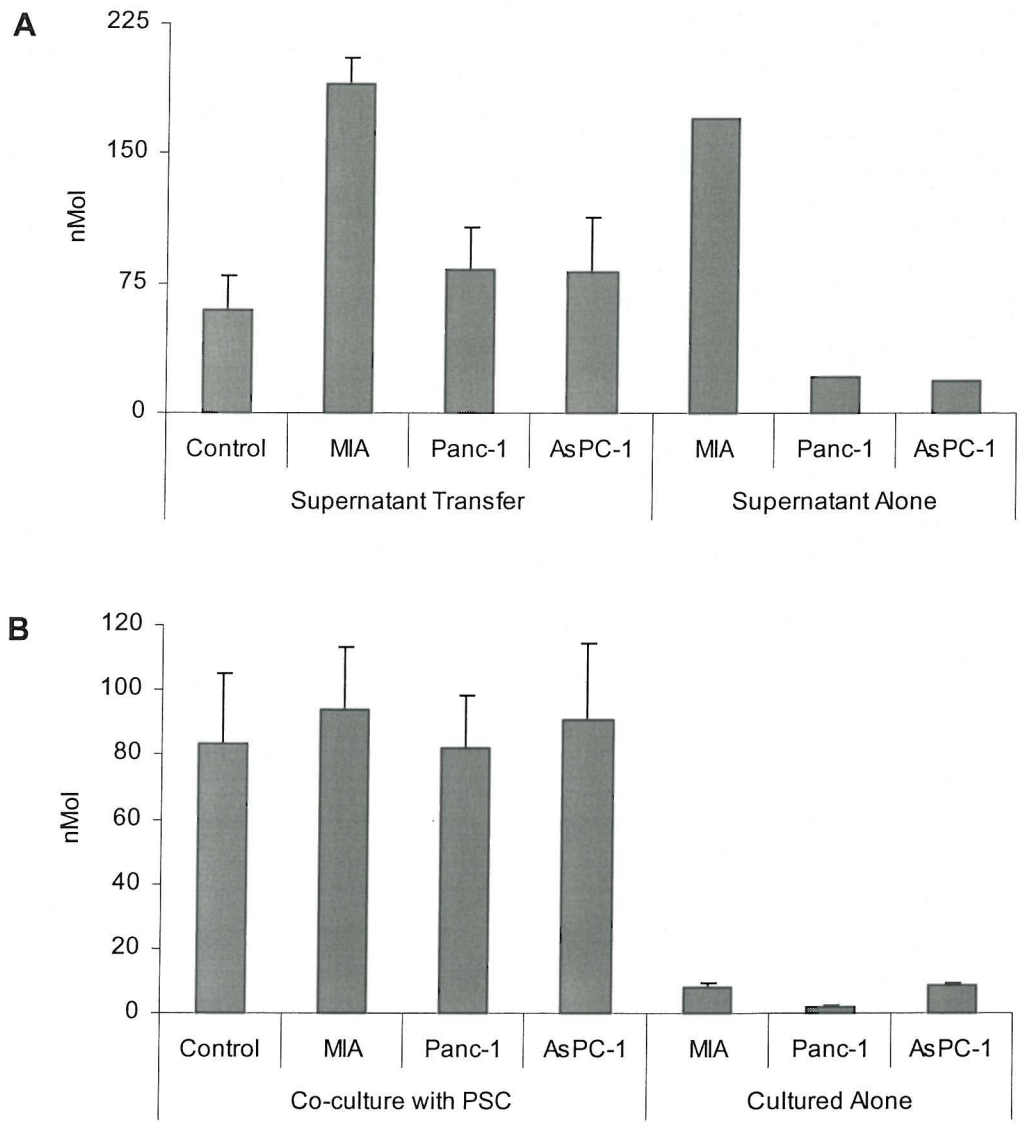


Figure 4.14
PSC TIMP-1 expression in supernatant transfer and co-culture models
TIMP-1 expression in supernatants from **A.** the supernatant transfer or **B.** co-culture models, measured using Biotrak ELISA. TIMP-1 expression in supernatants of cancer cells and PSC cultured alone ('control') are also shown. Bars represent the mean of 3 independent experiments +/-SEM. All experiments were conducted in 0.5%FCS, which contained negligible quantities of TIMP-1 (38ng/ml or 1.3nM).

These assays suggest TIMP-1 is secreted by PSC in excess of that by cancer cells. There is a discrepancy in the TIMP-1 detected in the supernatant derived from MIA PaCa-2 conditioned media and the controls for the co-culture models. This may be related to different culture techniques between models. The supernatant was made from sub-confluent cells in large flasks, compared to the relatively few cells used in the co-culture experiments. The results were again normalised to DNA, which ensured that changes in proliferation were not masking regulatory changes in TIMP-1 (Fig 4.15).

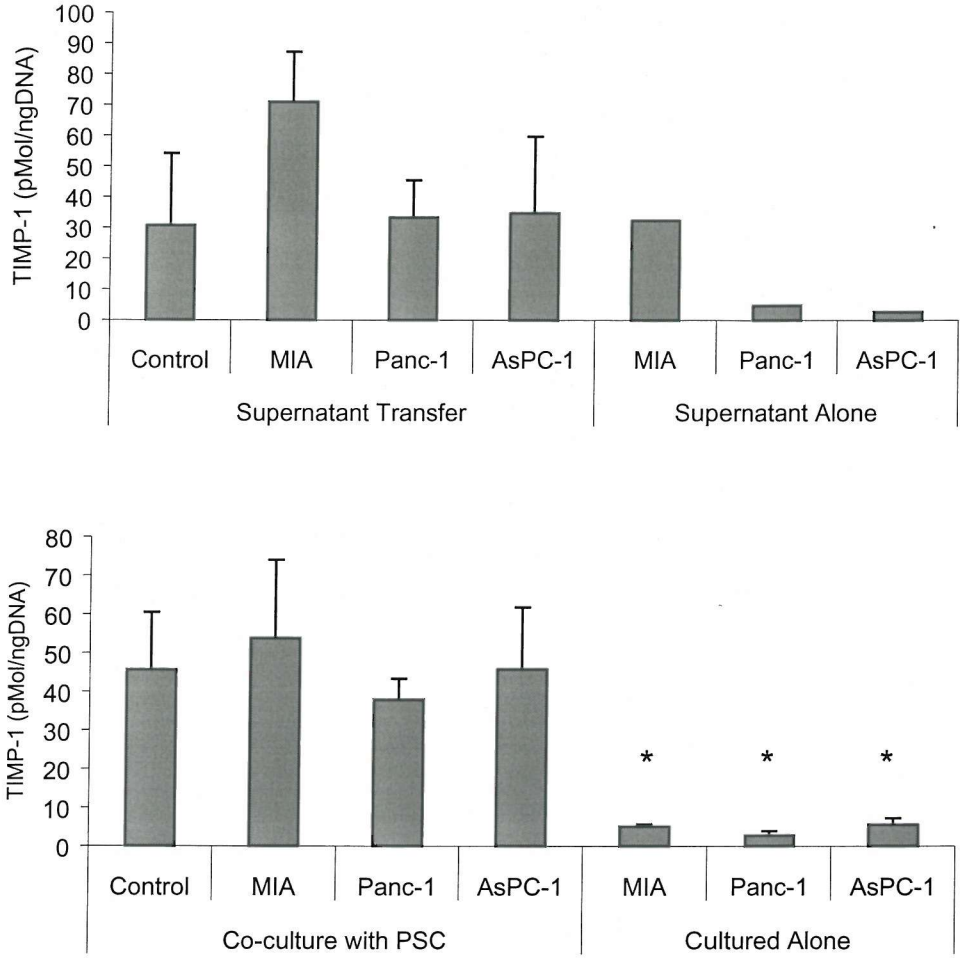


Figure 4.15
PSC TIMP-1 expression in supernatant transfer and co-culture models normalised to DNA
TIMP-1 expression in supernatants from **A.** the supernatant transfer or **B.** co-culture models, measured using Biotrak ELISA. TIMP-1 expression in supernatants of cancer cells and PSC cultured alone ('control') are also shown. Bars represent the mean of 3 independent experiments +/-SEM, * $p<0.05$ with respect to control. The results were normalised for DNA using the equations listed in the methods.

Taken together, the study of gelatinase and TIMP-1 expression in tissue culture models of pancreatic cancer have led to a series of findings that complement other data outlined in this chapter:

1. PSC expressed MMP-2 at between 7.2 and 27.8 the per cell equivalent of pancreatic cancer cell lines. Between 1.5-6% was endogenously active (data from co-culture model).
2. PSC expressed TIMP-1 at 8.1-16.6 fold higher concentrations than the pancreatic cancer cells (data from co-culture model).
3. There was no consistent evidence that the pancreatic cancer cell lines tested in these experimental models regulated MMP-2 or TIMP-1 expression in human PSC.
4. TIMP-1 was expressed at approximately 100 fold the concentration of MMP-2.

These features will be discussed in the context of the malignant phenotype.

Discussion

Results from this series of experiments provide evidence of a mechanism by which the stromal compartment of pancreatic ductal adenocarcinoma may expand to form the gross desmoplastic reaction that is observed *in vivo*. In the context of what is already known about stellate cell biology, these findings represent an important advance.

In health, stellate cells exist in a quiescent state, with one of their functions being to store retinoids. It is well recognised that during culture *in vitro*, stellate cells become activated, developing a myofibroblastic phenotype and this was confirmed in the cultured human PSC used in these experiments by demonstration of strong αSMA expression (Fig. 2.1). Evidence has recently emerged indicating the activation process can be propagated by conditioned media from MIA PaCa-2 and Panc-1 cells in rat PSCs (Park et al., 2002). It was not possible to test this in human PSC because of the relatively limited numbers isolated.

Once activated, the myofibroblastic stellate cell has a phentoype characterised by proliferation, matrix secretion and TIMP secretion. This leads to progressive fibrosis characterised by fibrillar collagen deposition until the injurious stimulus is removed. Stellate cell mediated fibrosis in the liver has been shown to be reversible and this was characterised by a reduction in stellate cell number through apoptosis (rather than reversion to a quiescent phenotype) (Iredale, 2001). There is no evidence at present that PSC mediated fibrosis in benign pancreatic disease (chronic pancreatitis) is

reversible. However, ductal adenocarcinoma is invariably progressive, therefore it is expected that the myofibroblastic phenotype of stellate cells is perpetuated.

Conditioned media from the pancreatic cancer cells promoted proliferation of cultured PSCs, presumably by paracrine stimulation. This observation provides a potential mechanism as to why numerous myofibroblastic PSC surround malignant glands in PDAC. Circumstantial evidence suggests that pancreatic cancer cells secrete growth factors capable of promoting cell division amongst PSC and these are shown in table 4.2 (below). Transfer of PSC conditioned medium to cancer cells led a modest increase in proliferation of AsPC-1 cells, but not the other cell lines. The interpretation of this data has to be limited because it was necessary to use rat PSC to condition the media (the irregular supply of human PSC often slowed progress in these experiments). It is worth noting the limited proliferative response by the cancer cell lines to 5%FCS, which is known to contain numerous growth factors. Pancreatic cancer cell lines can actively proliferate in low serum and serum free conditions (Murphy et al., 2001). This indicates that they are likely to be subject to powerful autocrine stimulation and that they are relatively growth factor independent as a result of oncogenic mutations (e.g. k-ras and p53) (Moore et al., 2001). It would be interesting to compare PSC proliferation after transfer of conditioned media from benign ductal epithelium with that from malignant cells. However, it is not possible to culture these cells *in vitro* for a variety of reasons including the fact that they secrete bicarbonate (Ulrich et al., 2002). Similar tissue culture models of hepatocellular carcinoma suggest that benign hepatocytes do not elicit the same mitogenic response from HSC as malignant hepatocytes (Nhieu et al., 1998). *In vivo* evidence in the pancreas indicates that stimulation of PSC growth does not occur because very few PSC were identified by immunohistochemistry in the normal pancreas. There is evidence of paracrine stimulation of myofibroblasts in and around neoplastic lesions in other organs. Increased numbers of myofibroblasts are present near cervical epithelial dysplasia/carcinoma in-situ and colonic adenomatous polyps (Schurch, 1999; Adegboyega et al., 2002). The myofibroblasts become much more numerous after invasion of the basement membrane, however, suggesting they are increasingly stimulated as the disease progresses (Yen et al., 2002; Tuxhorn et al., 2001; Noel and Foidart, 1998; Martin et al., 1996). The next logical step in pancreatic cancer research would be to study αSMA expression in PanIN lesions to determine when the PSC population becomes stimulated.

PSC growth factors/cyokines			Growth Factors over-expressed in PDAC
Activation	Mitosis	ECM synthesis	
TGFβ		TGFβ	TGFβ
TNFα	TNFα		
Il-6			
Il-1			
Il-10		Il-10	
	PDGF	PDGF	PDGF
	FGF	FGF	FGF
	?CTGF		CTGF
		TGFα	TGFα
			EGF
			VEGF

Table 4.2
Growth factors and cytokines that may be important in the interactions between pancreatic cancer cells and PSC
This was compiled from literature referenced in Chapter 1.

Increased numbers of PSC would in itself support increased collagen synthesis in the desmoplastic reaction but pancreatic cancer is known to be rife with TGFβ and it was therefore important to determine if collagen synthesis was also up-regulated in PSC (Friess et al., 1993). Pancreatic cancer cell lines have previously been shown to contain mRNA for extracellular matrix components but studies *in vivo* have clearly localised procollagen I and III mRNA and procollagen I to stromal cells (co-localising with αSMA staining) (Lohr et al., 1994; Gress et al., 1995; Yen et al., 2002). The collagen secreting phenotype of PSC was confirmed *in vitro* by demonstrating PSC secreted 4.8-8.3 fold more collagen than the cancer cells lines.

Collagen synthesis can be subject to transcriptional and post-transcriptional regulation and the fate of secreted collagen is controlled by secretion of MMPs and TIMPs (Lindquist et al., 2000; Nagase and Woessner, Jr., 1999; Gomez et al., 1997). The regulation of collagen metabolism in pancreatic cancer was carefully planned to examine each of these areas in turn. Conditioned media from 2 of the 3 pancreatic cancer cell lines increased human PSC procollagen I mRNA (although not significantly so). This was consistent with other pilot studies, which suggested conditioned media from cancer cells upregulated PSC procollagen I as well as procollagen III and fibronectin mRNA expression (Bachem et al., 2000). Media from AsPC-1 cells also increased collagen synthesis at protein level but this was not the case with media from Panc-1 cells, the reason for which is not clear. This inability to stimulate collagen

synthesis is consistent with the observation that Panc-1 cells induce very little stromal reaction after orthotopic implantation in nude mice, compared to AsPC-1 cells (Lohr et al., 2001). In that study it was concluded that the relative ability of pancreatic cells to induce desmoplasia stemmed from relative expression of TGF-β_1 and FGFs (measured at RNA level), however TGFβ_1 was expressed at fairly constant level by both cell lines, when secreted TGFβ_1 was measured directly (Lohr et al., 2001). MIA PaCa-2 conditioned medium contained very low levels of TGF-β_1 and it did not stimulate procollagen I gene expression or collagen synthesis by PSC. Hence pancreatic cancer cells have the potential to increase PSC collagen synthesis but it is likely that the consistent stimulus to PSC proliferation is quantitatively the more important event in deposition of collagen in the desmoplastic reaction. The fate of collagen once synthesised appears to be largely under the control of the stellate cell.

Amongst PSC supernatants TIMP-1 was detected at approximately 200 times the concentration of MMP-2, which even allowing for some variation in absolute measurements between the proprietary MMP-2 and TIMP-1 kits represents an important finding. This differential expression is entirely consistent with a balance that clearly favours accumulation of extracellular matrix *in vivo*. TIMP-1 mRNA has been consistently demonstrated in pancreatic cancers by Northern analysis of whole tissue lysates. In situ-hybridisation has localised transcripts to both stromal and malignant epithelial cells, although one immunohistochemical analysis suggested TIMP-1 was predominantly found in the malignant cells (Gress et al., 1995; Bramhall et al., 1997; Bramhall et al., 1996). However, MIA PaCa-2 cells appeared to be the only cell line to express TIMP-1 at levels comparable to PSC *in vitro*. There was no evidence from this data to suggest that the cancer cells regulate PSC TIMP-1 expression. In the context of tumour biology, induced over-expression of TIMP-1 in pancreatic cancer cells has been shown to reduce invasion of pancreatic cancer cells and also reduce tumour implantation, growth and metastasis in vivo, culminating in longer patient survival (Rigg and Lemoine, 2001; Bloomston et al., 2002b). It is interesting to note that high TIMP-1 expression in the tumours or the serum of patients suffering from oesophageal, gastric, colonic, lung and breast cancer is a bad prognostic indicator (Schrohl et al., 2003; Mori et al., 2000; Ylisirnio et al., 2000; Yoshikawa et al., 2000; Joo et al., 1999). Furthermore, there is accumulating evidence to suggest that TIMP-1 may have growth promoting properties to a variety of cell types including lymphoma cells, breast epithelial cells and hepatic stellate cells (Guedez et al., 1998; Hewitt et al., 2000; Murphy et al., 2002). This evidence taken together with the failure of MMP inhibitors to confer any therapeutic benefit in the treatment of pancreatic cancer suggest that the

role of TIMP-1 in cancer is likely to be very complex (Bloomston et al., 2002a). Another extracellular protease inhibitor, SERPINE (a serine protease inhibitor with activity against thrombin, trypsin, plasmin, urokinase plasminogen activator and other serine proteases) has also been shown to be important in pancreatic cancer. Expression of SERPINE leads to a phenotype characterised by induction of desmoplasia and invasion (Buchholz et al., 2003).

MMP-2 was studied in these experiments because its expression correlates with venous invasion, metastasis and a worse prognosis in pancreatic cancer (Matsuyama et al., 2002; Nagakawa et al., 2002; Koshiba et al., 1998). It was therefore concluded to provide the best representative of MMPs in pancreatic cancer to study *in vitro*. MMP-2 was expressed at significantly higher levels by PSC compared to pancreatic cancer cells when measured by both zymography and MMP-2 assay, thus indicating that PSC may be an important source of MMP-2 *in vivo*. MMP-2 is readily detected in pancreatic cancer by Northern analysis and localised to stromal cells and malignant epithelium by in-situ hybridisation and immunohistochemistry (Gress et al., 1995; Bramhall et al., 1996; Bramhall et al., 1997). It is possible that MMP-2 expression amongst pancreatic cancer cell lines was under represented *in vitro* (Ellenrieder et al., 2000). There is a recognised discrepancy between the inconsistent MMP-2 expression by pancreatic cancer cell lines *in vitro* and consistent MMP-2 expression by malignant epithelium *in vivo*. Orthotopic implantation of pancreatic cancer cells in nude mice significantly increased their MMP-2 expression and pancreatic cancer homogenates subjected to zymography had a greater active fraction of MMP-2 compared to homogenates of normal pancreas(Haq et al., 2000) (Koshiba et al., 1998). This suggests that there may be an interaction *in vivo* promoting MMP-2 expression and activation within pancreatic cancer. With respect to interactions between cancer cells and PSC *in vitro*, there was no evidence that the cancer cells up-regulated either MMP-2 expression or activation levels in PSC. Although MMP-2 expression by PSC was greater than the cancer cells, total activated MMP-2 was equivalent (Fig. 4.8).

Despite the identification of abundant mRNA for MMP-9 in both stromal and malignant cells *in vivo* it was barely measurable *in vitro*, in either PSC or cancer cells, which is consistent with previous findings (Ellenrieder et al., 2000; Phillips et al., 2003). The zymograms indicate that the active fraction of MMP-9 is greater in the PSC but again there was no evidence of regulation by cancer cells in these cultures.

Conclusions

In these tissue culture models there was evidence that the interaction between cancer cells and PSC has the potential to promote development of the desmoplastic reaction in pancreatic cancer, as a result of stellate cell proliferation and collagen synthesis, which is promoted by cancer cells. Furthermore, matrix accumulation would be supported by abundant TIMP-1 inhibiting proteolytic activity of MMPs and therefore favouring matrix accumulation

Chapter 5

Role of desmoplastic extracellular matrix in pancreatic cancer cell growth

Introduction

Early in the investigation of the overall hypothesis, it was noticed that type I collagen increased ^{3}H-thymidine incorporation amongst the pancreatic cancer cell lines. This was a key observation because PSC may indirectly promote the malignant phenotype of pancreatic cancer *in vivo*, by secreting extracellular matrix that in turn supports growth of the malignant cells. Two key mechanisms that control the number of cells in a population: cell proliferation and apoptosis and an imbalance between these mechanisms often exists in malignancy. The influence of collagen (and other extracellular matrix) on the balance of proliferation and apoptosis in pancreatic cancer was investigated in the next two chapters.

Hypothesis

The alteration of the quality of extracellular matrix, characterised by a relative excess of type I collagen in the desmoplastic reaction (compared to the normal pancreas), supports growth of pancreatic cancer.

Aims

To investigate how collagen and other matrix components found in the desmoplastic reaction influence cancer cell proliferation.

Methods

Proliferation was quantified indirectly by measuring DNA synthesis with ^{3}H-thymidine incorporation. The effect of extracellular matrix on growth of the cancer cells was also studied with Fluorescence Activated Cell Sorter (FACS) analysis.

1. ^{3}H-thymidine incorporation

Experiments studying the effects of extracellular matrix on the growth of the cancer cells were undertaken using the methods described in Chapter 2. All results were normalised to DNA (measured using PicoGreen) to control for the inevitable variation in adhesion of the cells to the different extracellular matrices (see appendix 5).

2. Analysis of cell cycle using FACS

Propidium Iodide (PI) Staining

Pancreatic cancer cells ($6x10^5$) were seeded in $25cm^2$ flasks prepared with collagen using protocols in Chapter 2. After 24 hours of culture, the cells were washed with HBSS+ and the medium changed to 0.5%FCS v/v DMEM, thereby recreating the conditions used in the ^{3}H-thymidine incorporation experiments. After 8 hours the cells were trypsinised and collected in a pellet by centrifugation. Cells were washed twice in PBS, then fixed/permeablised in 70% ethanol for 1 hour at 4°C. After this step the cells were resuspended in PBS with 5mg/ml PI and 2mg/ml ribonuclease A (both Sigma). Degradation of RNA with ribonuclease meant that PI stained only DNA. After 30 minutes incubation in the dark, the cells were analysed. Data was collected on channel FL-2 of the FACS machine (Becton Dickinson) and a dot plot generated by plotting FL2-A (area) against FL2-W (width) thus generating a dot plot (Fig. 5.1).

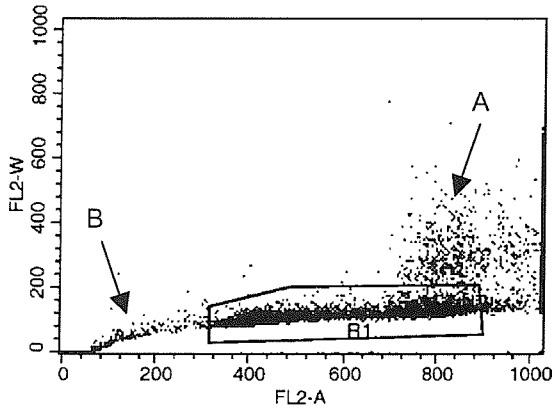


Figure 5.1
Dot plot generated after FACS analysis of cells stained with PI
Events were acquired of channel FL2 and then plotted according to area (FL2-A) and width (FL2-W) thus allowing exclusion of doublets (A) and apoptotic cells (B) by gating the population of cells to be studied (R1).

Firstly this analysis allowed cells to be separated by their DNA content (i.e. 2n, 4n etc). Secondly, doublets could be identified (and excluded, A Fig. 5.1) by virtue of presenting a greater width to the laser in the FACS machine. Finally, apoptotic cells could be excluded from analysis by identifying DNA content <2n (B Fig. 5.1). Exclusion of these cells was achieved by gating the cell population with a normal width and 2n-4n DNA content, using CellQuest Software (Becton Dickinson). Histograms were then generated from the gated population of cells, allowing comparison of the cell cycle profile of cells on different extracellular matrix.

Bromodeoxyuridine (BrdU) incorporation

BrdU is a thymidine analogue that is readily incorporated into DNA of cells in S phase. FACS was employed to identify incorporated BrdU that was subsequently stained with fluorescein isothiocyanate (FITC) conjugated antibodies. The method is published on the Cancer Research UK website (www.icnet.uk). $2x10^7$ cells were seeded into $75cm^2$ flasks prepared with collagen as previously described. After 24 hours culture in normal media, cells were washed with HBSS+ and the medium replaced with 0.5%FCS v/v DMEM. Cells were pulsed with 25mM BrdU (Becton Dickinson) for 1-6 hours, then harvested immediately or returned to culture in medium free of BrdU and harvested later (pulse chase experiments). Cells were harvested using trypsin, fixed with 70% ethanol, washed in PBS then exposed to 2M HCl, which denatured cellular DNA thus exposing incorporated BrdU. The acid was thoroughly removed by washing with PBS and PBST (0.1% BSA/0.02% Tween 20 in PBS) and BrdU labelled with anti-BrdU primary antibodies/ species specific FITC conjugated secondary antibodies (M0744/F0479 Dako). After further washes, the cells were counterstained with PI/ribonuclease using the method described above. Cells were sorted by virtue of FITC staining in channel FL1-H (log scale) and PI staining in channel FL2-H.

3. Experiments using β_1 integrin blocking antibodies

The role of β_1 integrins in pancreatic cancer cell adhesion and proliferation was investigated using a mouse anti-human β_1 integrin blocking antibody, clone 4B4 (Beckman Coulter).

Adhesion Assay

An adhesion assay was undertaken to determine if this antibody would block adhesion of pancreatic cancer cells (Sethi et al., 1999). $5x10^4$ cancer cells were incubated with β_1 integrin blocking antibody (0-10µg/ml) for 15 minutes, then seeded into the wells (prepared with collagen as previously described). After 45 minutes incubation at 37°C, non-adherent cells were washed away with PBS (x3) and the remaining adherent cells fixed with 3% formaldehyde and subsequently stained with 1% methylene blue (Sigma) w/v dH_2O. After 15 minutes excess methylene blue was removed by washing with dH_2O (x4) and the stained cells dissolved in 0.5M HCl. The optical density was read at 620nm on a spectrophotometer. On one occasion the experiment was undertaken in parallel using isotype non-immune IgG1 (Sigma), which had no effect on cellular adhesion in this assay.

^{3}H-thymidine Incorporation Assay

The effect of the β_1 integrin blocking antibody on growth of the cancer cells was also examined. Pancreatic cancer cells were incubated with the β_1 integrin blocking antibody (for 15 minutes) then seeded onto TCP and collagen types I and IV. After 24 hours, the medium was changed to 0.5%FCS v/v DMEM, thus removing excess antibody and recreating the experimental conditions of earlier experiments. After a further 8 hours ^{3}H-thymidine incorporation was measured amongst the cell cultures according to methods described in Chapter 2 (including normalising results to DNA). Experiments were conducted in parallel (on each repetition) using isotype non-immune IgG1 (Sigma).

4. Experiments using collagen derived peptides

The biological effect of a peptide derived from the non-collagenous domain (NC1) of the α_3 chain of type IV collagen (sequence CNYYSNSYSFWLASLNPER (Clonestar)) was tested, with a view to understanding differences that were observed in growth regulation by type I and type IV collagen (see results)(Han et al., 1997). $1x10^4$ pancreatic cancer cells were seeded directly onto TCP in 24 well plates and incubated in normal medium for 24 hours. At this stage the medium was replaced (after washing the cells layer in HBSS+) with 0.5%FCS v/v DMEM containing the α_3(NC1) peptide (5-250μg/ml). A nonsense peptide of similar size (sequence LKRKGLVKKVQAFLAECDTVE (Clonestar)) was used as a control in these assays. ^{3}H-thymidine incorporation was subsequently measured over the following 16 hours, using the method described in Chapter 2. [N.B. Both peptides were initially suspended in DMSO (by necessity) but final concentration of DMSO in the assays was 0.5% by volume and its presence did not alter background scintillation counts].

5. Method for coating wells with pancreatic stellate cell derived matrix

The desmoplastic reaction contains a variety of extracellular matrix and in order to determine if PSC derived matrix would regulate the growth of cancer cells *in vitro*, a method described by Kouniavsky et al. was adopted (Kouniavsky et al., 2002). $2.5x10^4$ human pancreatic stellate cells were seeded into wells of a 24 well plate. They were left until a confluent monolayer had formed, usually within 5 days. The cells were removed using 0.5% triton X and wells washed 3 times with PBS. There were no signs of cell debris left in the wells, by light microscopy, after completion of this process. In keeping with other experiments using extracellular matrix, the PSC

derived matrix was blocked using BSA. $1x10^4$ cancer cells were seeded into triplicate wells for measurement of ^{3}H-thymidine incorporation (with duplicate wells for PicoGreen analysis). This was undertaken using methods described in Chapter 2.

Results

Tissue culture plastic (TCP) was used as a control in all experiments using extra-cellular matrix and throughout these experiments results have been normalised to results obtained on TCP to control for inter-experimental variation.

1. Effect of collagen on pancreatic cancer cell morphology

Pilot experiments indicated that collagen regulated growth of pancreatic cancer cells and it also altered their morphology (Fig. 5.2). Panc-1 and AsPC-1 cells became spread out and grew individually on type I collagen, compared to small clusters of cells that were observed on plastic. In contrast MIA PaCa-2 grew in clusters and displayed a pyknotic morphology on type I and IV collagen compared to TCP. Cells cultured on type IV collagen were morphologically indistinguishable from cells cultured on type I collagen.

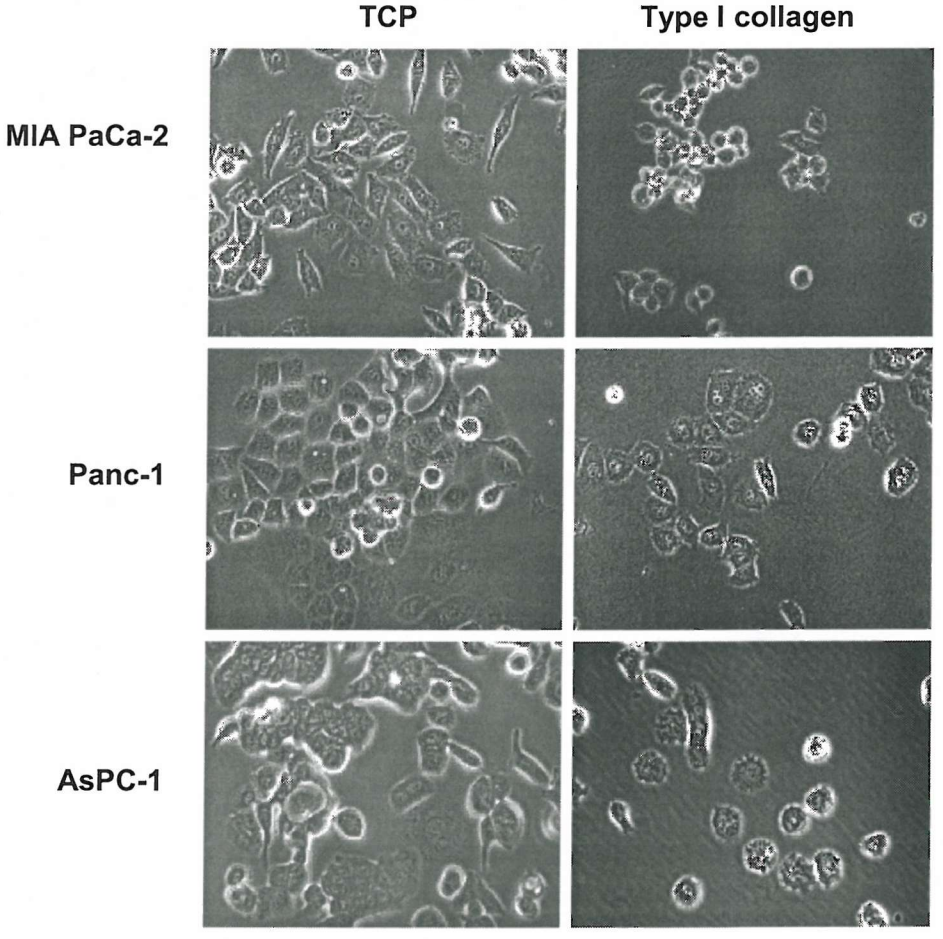


Figure 5.2
Morphological appearance of pancreatic cancer cells cultured on collagen
Photomicrographs (magnification (x200) demonstrating morphological appearances of cancer cells cultured on type I collagen for 24 hours.

Panc-1 and AsPC-1 cells attached rapidly to type I collagen, as shown by measuring the number of adherent cells after 45 minutes (Fig. 5.3). MIA PaCa-2 cells took much longer to adhere to collagen types I and IV and after 24 hours approximately 50% of the cells remained unattached (Fig. 5.3).

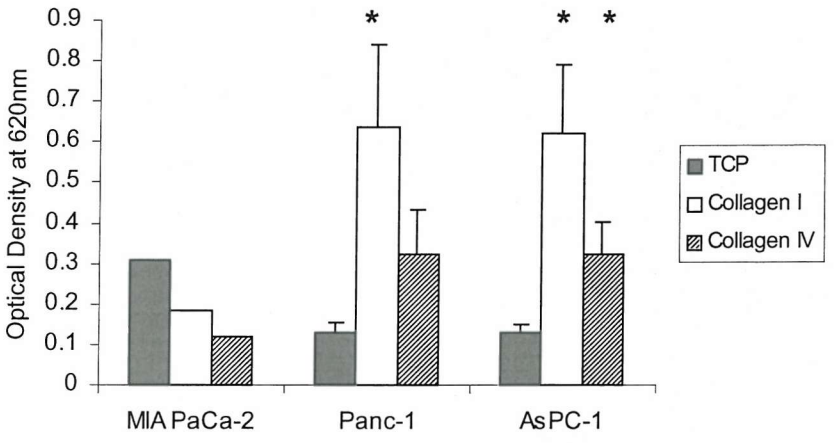


Figure 5.3
Effect of collagen on the attachment of pancreatic cancer cells in cell culture
$5x10^4$ cells were seeded onto TCP or collagen types I or IV and incubated at 37°C for 45 minutes (90 minutes for MIA PaCa-2). The number of cells was then quantified by staining them with methylene blue and measuring the optical density at 620nm as described in the methods of this chapter. Bars represent the mean of 3 separate experiments (except MIA PaCa-2 where n=1). (*$p<0.05$ with respect to TCP for each cell line).

2. The effect of collagen on growth of pancreatic cancer cells

A series of experiments were undertaken with proprietary, purified extracellular matrices to study growth of the pancreatic cancer cells when in contact with various components of the desmoplastic reaction. Collagen, particularly collagen types I and III, comprise much of the extracellular matrix encountered in the desmoplastic reaction. After observations in pilot experiments that indicated type I collagen altered growth of pancreatic cancer cells, experiments were undertaken to determine if coating density of collagen would alter ^{3}H-thymidine incorporation by the cancer cells. Accordingly, collagen was serially diluted with acetic acid and equal volumes were used to coat wells in 24 well plates, using the protocols outlined in Chapter 2. This demonstrated ^{3}H-thymidine incorporation increased with the coating density of collagen, in all cell lines (Fig 5.4). Based on these results, future protocols employed a coating density of 15μg/cm^2 for collagen. Type IV collagen was introduced to the study at a later date and used empirically at a coating density of 15μg/cm^2 to ensure consistency.

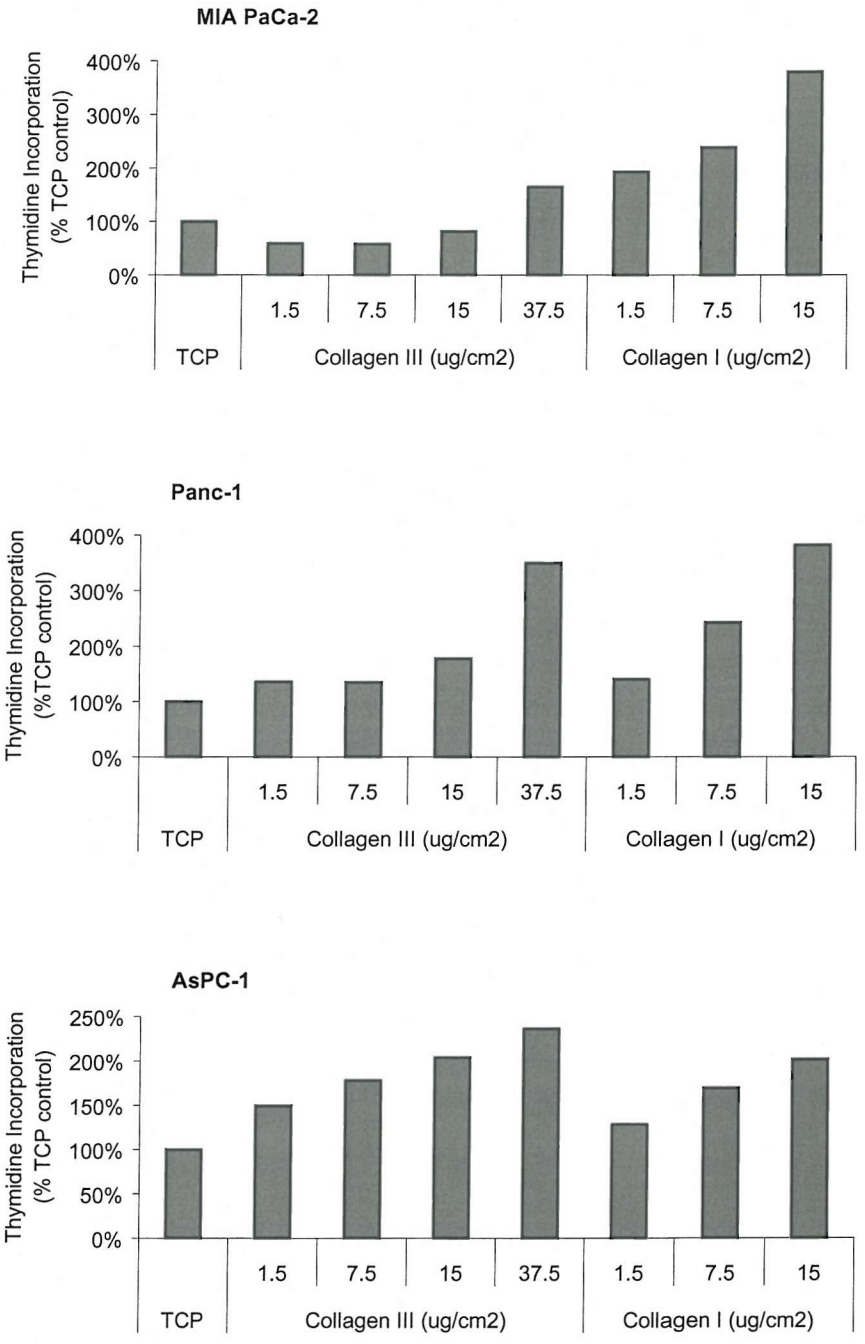
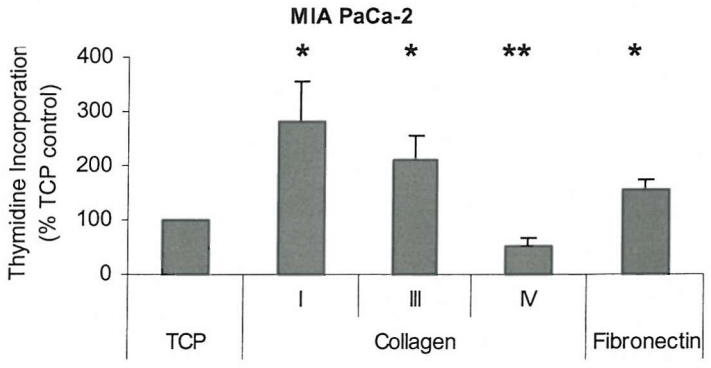


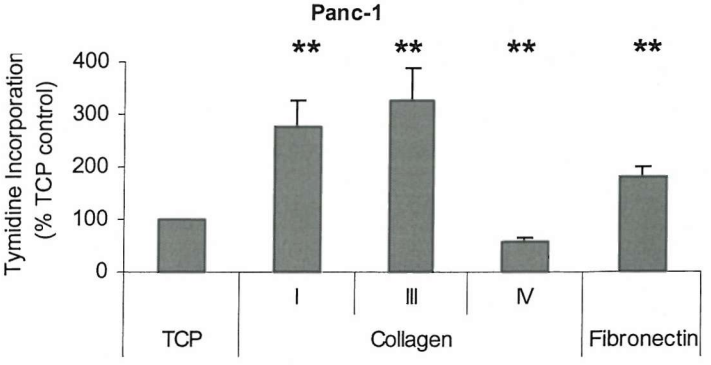
Figure 5.4
Effect of coating density of collagen on ^{3}H-thymidine incorporation by pancreatic cancer cell lines
$1x10^{4}$ pancreatic cancer cells were seeded onto collagen I and III coated at different densities. After 24 hours the medium was changed to 0.5%FCS and ^{3}H-thymidine incorporation subsequently measured over 16 hours. Bars represent the mean of a triplicate from a single experiment.

Having established a good tissue culture model, the effect of type I and III collagen (fibrillar, interstitial collagen) and type IV collagen (non-fibrillar, basement membrane collagen) had on growth of the pancreatic cancer cells was compared by

measuring ^{3}H-thymidine incorporation, normalised to total DNA (Fig. 5.5). This allowed a per cell measurement of proliferation rather than measuring proliferation of the whole population, which would have relied on the assumption that cells adhered to the different matrices equally. In contrast to adhesion in the short term-assays (Fig. 5.3), after 24 hours the number of adherent cells on TCP, types I and IV collagen were much more equal and correction for DNA did not substantially alter the results (this is discussed in more detail in appendix 5).

Fibrillar collagen consistently stimulated ^{3}H-thymidine incorporation in all the cancer cells compared to TCP. In contrast, type IV collagen was relatively inhibitory to ^{3}H-thymidine incorporation in MIA PaCa-2 and Panc-1cells (originally derived from primary tumours) compared to TCP. In each case, type IV collagen was relatively inhibitory to proliferation compared to collagen types I and III, although the differential effect of the collagen was much less marked in the metastatic AsPC-1 cells (Fig. 5.5). The pancreatic cancer cells were also cultured on fibronectin and the results are included to represent a 'neutral' extracellular matrix component. Fibronectin is a ubiquitous protein that exists in the serum and extracellular matrix. It is present in the desmoplastic reaction to a varying extent and all cells are exposed to it whether epithelial or mesenchymal in origin (Mollenhauer et al., 1987).





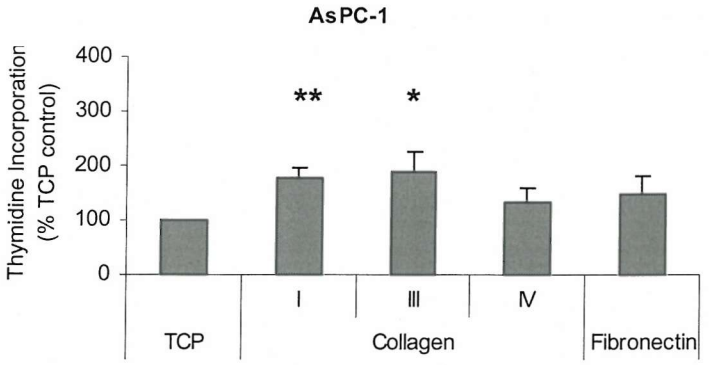


Figure 5.5
Comparative effect of collagen on ^{3}H-thymidine incorporation by pancreatic cancer cell lines
1x10^{4} pancreatic cancer cells were seeded onto collagen I, III, IV and fibronectin. After 24 hours the medium was changed to 0.5%FCS and ^{3}H-thymidine incorporation subsequently measured over 16 hours. Results were normalised to DNA giving final results in units of cpm/ngDNA, which were normalised to results obtained on TCP.. Bars represent the mean of 6 or more independent experiments +/-SEM, *p<0.05 and **p<0.01, with respect to TCP. ^{3}H-thymidine incorporation by MIA PaCa-2/ Panc-1 cultured on collagen types I and III was significantly greater than in cells cultured on collagen type IV. Collagen did not alter background scintillation counts. Between independent experiments the range of values in absolute cpm/ngDNA amongst the controls was MIA PaCa-2: 0.17-1.22, Panc-1: 0.15-1.62 and AsPC-1: 1.57-4.33).

The pattern of ^{3}H-thymidine incorporation in these tissue culture models was perpetuated with time (Fig. 5.6).

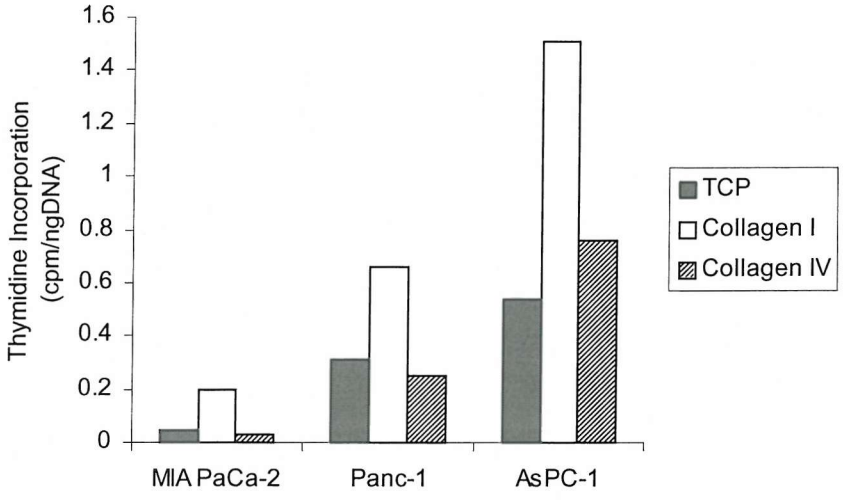


Figure 5.6
Effect of prolonged culture of pancreatic cancer cells lines on collagen
1x10^{4} pancreatic cancer cells were seeded onto TCP and collagen I or IV. After 72 hours the medium was changed to 0.5%FCS and ^{3}H-thymidine incorporation subsequently measured over 16 hours. Bars represent the mean of a triplicate

results from a single experiment. Results were normalised to DNA giving final results in units of cpm/ngDNA.

Through expression of MMPs and other proteases, the extracellular matrix surrounding cells is constantly subject to degradation and remodelling. It was possible to determine the effect of peri-cellular proteolysis, by culturing cells on r/r type I collagen, a mutant collagen resistant to proteolytic degradation (Fig. 5.7). This suggested that the pancreatic cancer cells did not derive the same degree of growth advantage from culture on non-degradable r/r collagen.

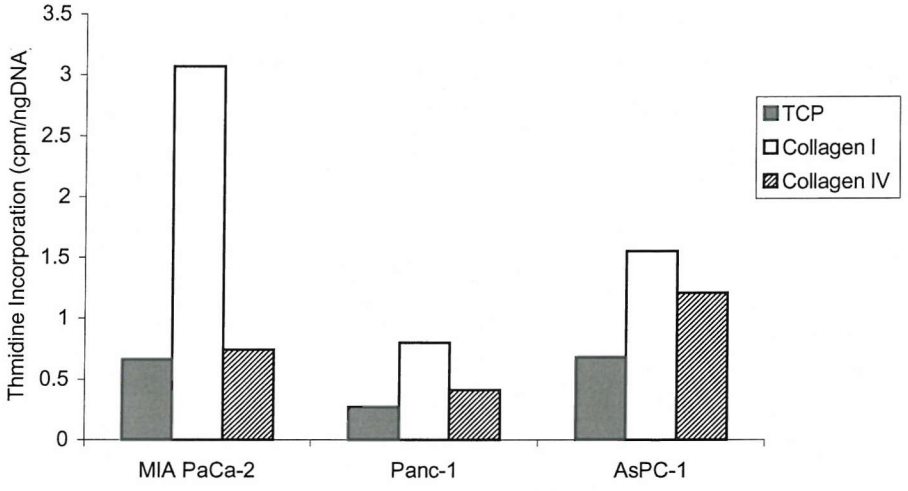


Figure 5.7
Comparison of wild type and r/r mutant type I collagen on the growth of pancreatic cancer cells
$1x10^4$ pancreatic cancer cells were seeded onto wild type or r/r mutant type I collagen. After 24 hours the medium was changed to 0.5%FCS and ^{3}H-thymidine incorporation subsequently measured over 16 hours. Results were corrected to DNA giving final results in units of cpm/ngDNA. The results for the wild type collagen are reproduced from figure 5.4 and results using the r/r mutant come from a single experiment (due to very limited availability of r/r collagen). These results have to be interpreted within the context of this limitation).

These results indicated that fibrillar collagens (types I and III) consistently supported proliferation of the pancreatic cancer cells, compared to TCP and type IV collagen, which were relatively inhibitory. The inhibitory effect of type IV collagen was less marked in the metastatic AsPC-1 cells. The effect of matrix on pancreatic cancer cell growth may be in part mediated by peri-cellular proteolysis.

Fluorescent Activated Cell Sorting (FACS) analysis was used to study the profile and kinetics of the cell cycle of pancreatic cancer cells cultured on different matrices. Initially it was used to determine if collagen altered the proportion of cells in different parts of the cell cycle. Pancreatic cancer cells were cultured on TCP and type I/IV collagen and after 24 hours the DNA was stained with propidium iodide (PI). The intensity of DNA staining correlated with total cellular DNA; ranging from 2n of a cell in G_1 to 4n of a cell just about to undergo mitosis, thus allowing crude division of cells into G_1, S and G_2M phases (Fig. 5.8).

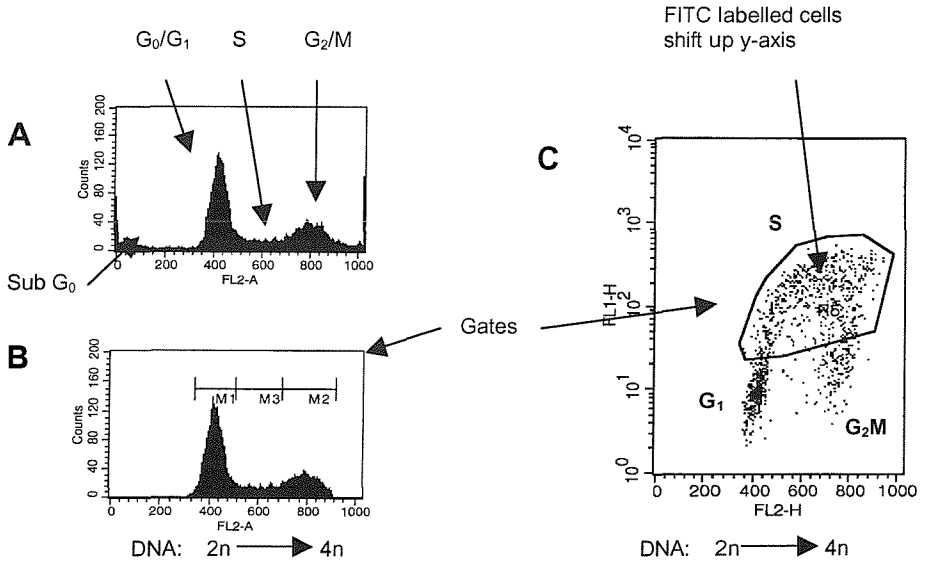


Figure 5.8
Principles of studying the cell cycle with FACS analysis
All data was analysed using CellQuest software (Becton Dickinson) **A.** MIA PaCa-2 cells stained with propidium iodide (PI), with phases of the cell cycle identified on the chart. The more DNA in a cell, the greater the intensity of the staining, thus shifting the cell right, along the x-axis. **B.** Standard gates applied to quantify cells in G_0, S and G_2M phases. **C.** Panc-1 cells exposed to a pulse of BrdU and subsequently labelled with FITC (using antibodies) and counterstained with PI. This allowed separation of cells by DNA content and FITC labelling, thus allowing cells in S phase to be distinguished from the remainder of the cells, permitting accurate quantification. Events identified <400 on the x-axis contain <2n DNA and are traditionally regarded as apoptotic. The raw data obtained from FACS analysis required the exclusion of apoptotic cells or doublets (see Fig. 5.1).

The PI stained cells provided a visual analogue of the profile of the cells cultured on TCP and type I/IV collagen. This did not indicate any shift of cells into cycle on type I collagen or cell cycle arrest on type IV collagen (Fig. 5.9).

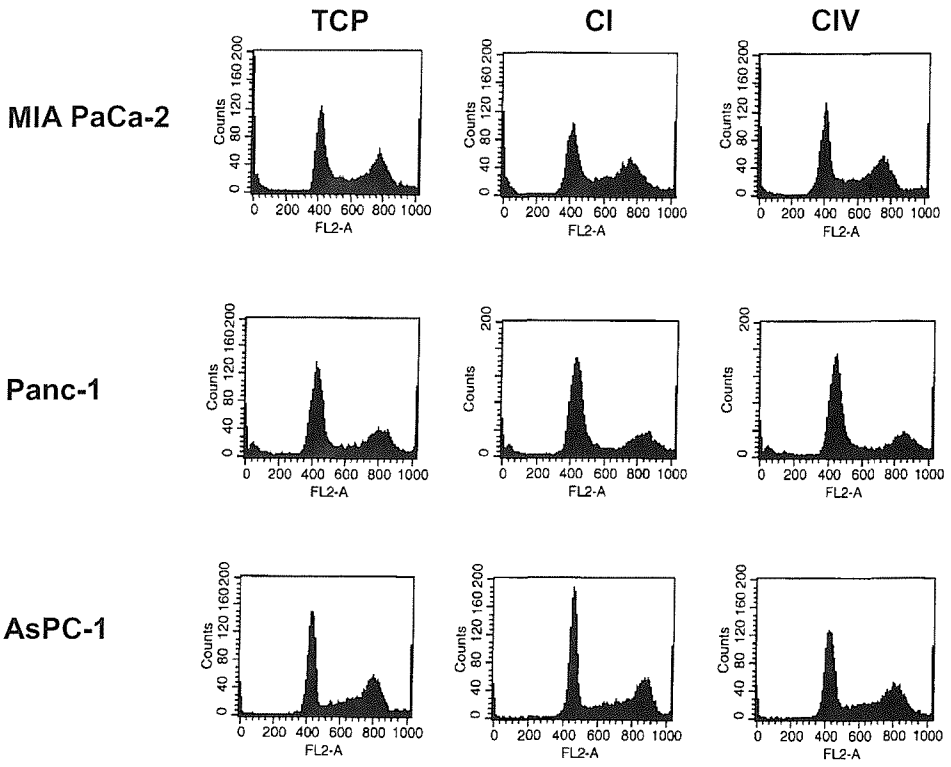


Figure 5.9
Histograms generated from FACS analysis of pancreatic cancer cell lines cultured on collagen
Pancreatic cancer cells were cultured on TCP or collagen types I or IV for 24 hours in 0.5%FCS prior to being stained with PI. Doublets were excluded from analysis by gating the population of single cells from a FL2 A vs FL2 W dot plot (see Fig. 5.1). Collagen did not obviously alter the DNA profile of the cells. Images representative of 2 separate experiments.

Gates corresponding to the various phases of the cell cycle were applied using (see Fig. 5.8) and the number of cells comprising the peaks in the histogram quantified (Fig. 5.10). This analysis confirmed what appeared to be the case from examining the histograms by eye: collagen did not alter the profile of cells in the cell cycle, with the exception of a non-significant change in MIA PaCa-2 cells. The proportion of MIA PaCa-2 cells increased slightly in G_1 phase, with a concomitant reduction of cells in G_2M phases, on collagen types I and IV.

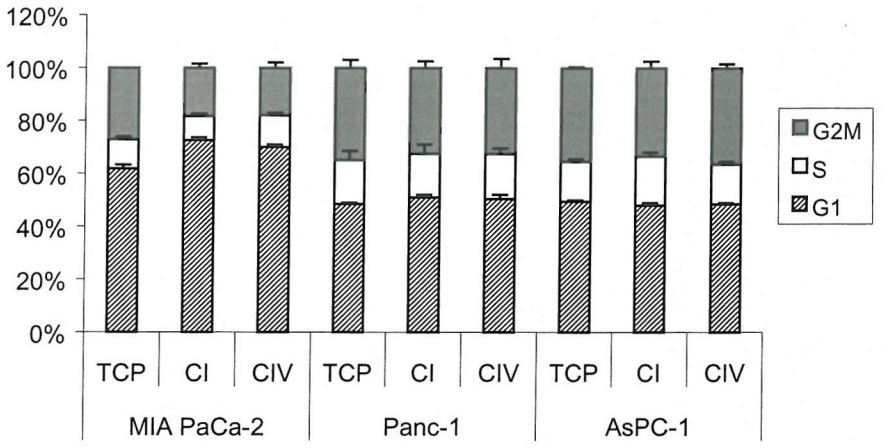


Figure 5.10
Quantification of fraction of cells within the cell cycle from the histograms generated from cancer cells stained with PI
Data comprising the bars comes from analysis of histograms of PI stained cancer cells, sorted by FACS, from 2 separate experiments (+/-SEM), in which the cancer cells were cultured on TCP, type I and type IV collagen (CI and CIV respectively). The bars show the relative proportion of cells in each phase of the cell cycle. The gates used are shown in Fig. 5.8.

Quantifying cells in different phases of the cell cycle using this method necessitated arbitrary gating, which does not account for overlap between cells in G_1/S phase or S/G_2M phase. To ensure subtle changes in cell cycle were not overlooked, a more sensitive technique was employed based on the identification of cells in S-phase with BrdU. Cells cultured on different matrices (as described previously) were exposed to BrdU and subsequently harvested. Incorporated BrdU was FITC labelled and the cells counterstained with PI. The FITC labelled BrdU effectively allowed the S phase cells to be distinguished from the other cells by virtue of their shift up the y-axis (see Fig. 5.8C). This permitted more accurate determination of the proportion of cells in the different phases of the cell cycle. The plots obtained are shown below (Fig 5.11).

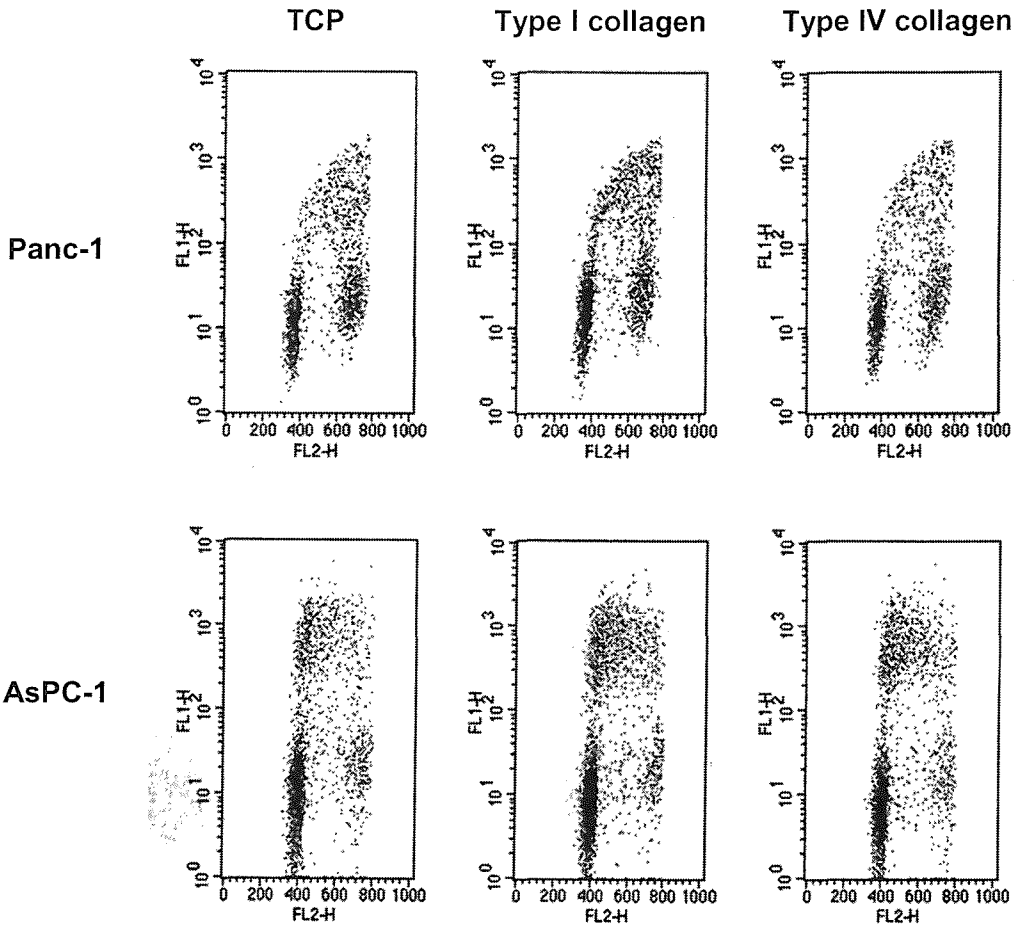


Figure 5.11
FACS analysis of pancreatic cancer cells exposed to BrdU, whilst cultured on TCP and types I/IV collagen
Dot plots of Panc-1 and AsPC-1 cells stained with PI (x-axis) and FITC labelled BrdU (y-axis) after culture of the cells on different matrices. Cells were exposed to BrdU for 2 hours. The cells fell into 3 groups: G_0 at 400 (2n) on the x-axis, G_2M at 800 (4n), with the s phase distinguished from cells in the other phase by virtue of their BrdU labelling (migrating up the y-axis following FITC labelling). These plots are representative of 4 separate experiments.

The experimental protocol used to label BrdU and counter stain with PI caused enormous loss of cells and the relatively inefficient adhesion of MIA PaCa-2 cells prevented any meaningful results being obtained with this cell line. Panc-1 and AsPC-1 cells, were pulsed with BrdU for 1, 2, 4 and 6 hours. A relative increase in the proportion of BrdU labelled cells was observed as more cells entered S phase over time but the relative fraction of cells in S phase for a given pulse of BrdU did not alter when the cells were cultured on collagen. This confirmed the findings of experiments employing PI staining alone and indicated that another mechanism must underlie the growth advantage derived from type I collagen.
These techniques were not dynamic, they provided a profile of the cell cycle at a single point in time. BrdU labelling was therefore used to study the kinetics of cells

cycling on the different matrices. Panc-1 and AsPC-1 cells were pulsed with BrdU for 1 hour, following which the medium was replaced (thus removing the BrdU) and the cells were subsequently harvested at increasing time intervals. This 'pulse-chase' type experiment labelled a cohort of cells in S phase, the progress of which was then followed through S and G_2M phase, over a period up to 21 hours. Sample dot plots obtained from these experiments are shown below (Fig. 5.12).

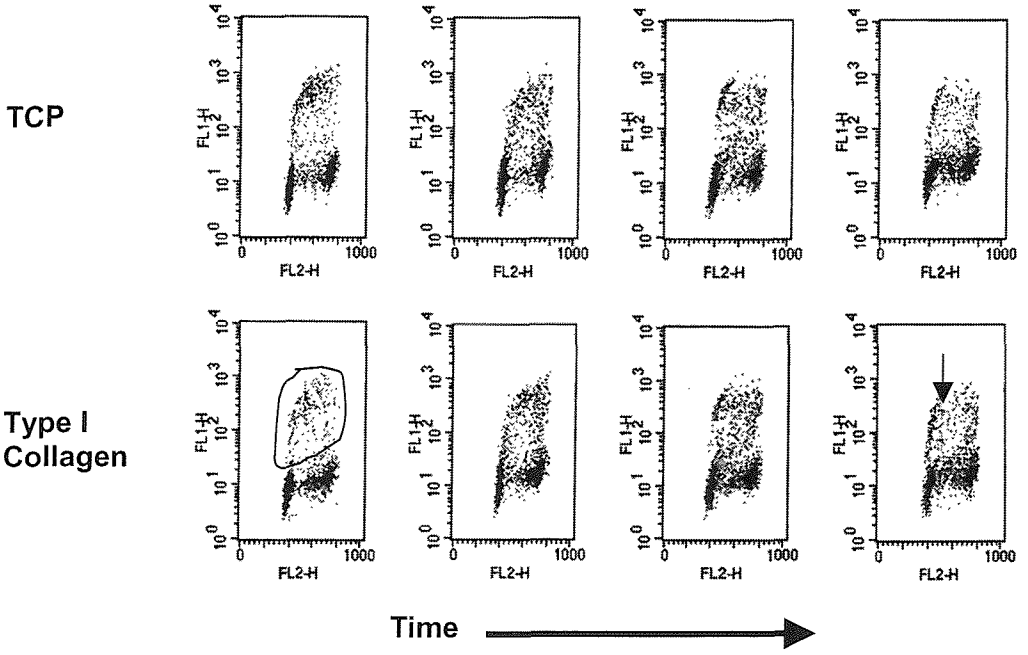


Figure 5.12
Example of dot plot acquired during pulse chase experiments
AsPC-1 cells cultured on TCP and type I collagen were pulsed with BrdU for 1 hour, then harvested either immediately or 7 hourly up to 21 hours. The BrdU labelled cells (FL1-H at $>10^2$) were observed moving through S phase as time progressed (i.e. increasing DNA content from 2n-4n) before undergoing mitosis and returning to 2n, where they were observed accumulating (arrow). The BrdU labelled cells from each time point were gated (as shown in bottom left dot plot) and histograms generated (Fig 5.13). This allowed a dynamic comparison of cell moving through the cell cycle when culture on TCP and type I collagen.

These experiments proved technically difficult and time consuming and were therefore confined to comparing TCP with type I collagen. Histograms generated from the BrdU labelled cells (Fig. 5.13) consistently demonstrated that a larger proportion of cells accumulated back in G_1 phase after 14-21 hours, on type I collagen compared to those cultured on TCP, implying a more rapid transition through S phase and/or G_2M phase (Fig. 5.13A and B).

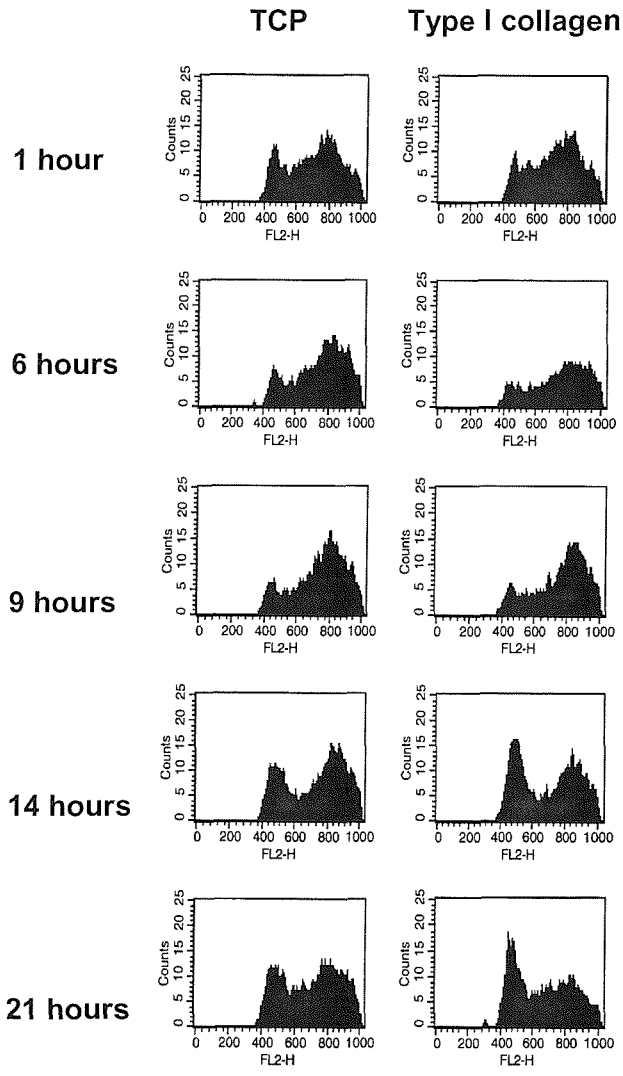


Figure 5.13A
Histograms demonstrating the results of BrdU 'pulse-chase' experiments in Panc-1 cells
Panc-1 cells were cultured on TCP or type I collagen using identical experimental protocols to those previously described. Interpretation of these histograms relied on studying their profile and not the absolute numbers. With time the labelled cells progressed through S phase and as the DNA content increased they shifted (right) along the x-axis, before undergoing cell division and shifting left as their DNA content halved (~400 on x-axis). After 14 hours the cells cultured on type I collagen are seen accumulating back at 2n (400) in greater numbers that those cultured on TCP and this became more pronounced at 21 hours. These images are representative of 3 separate experiments.

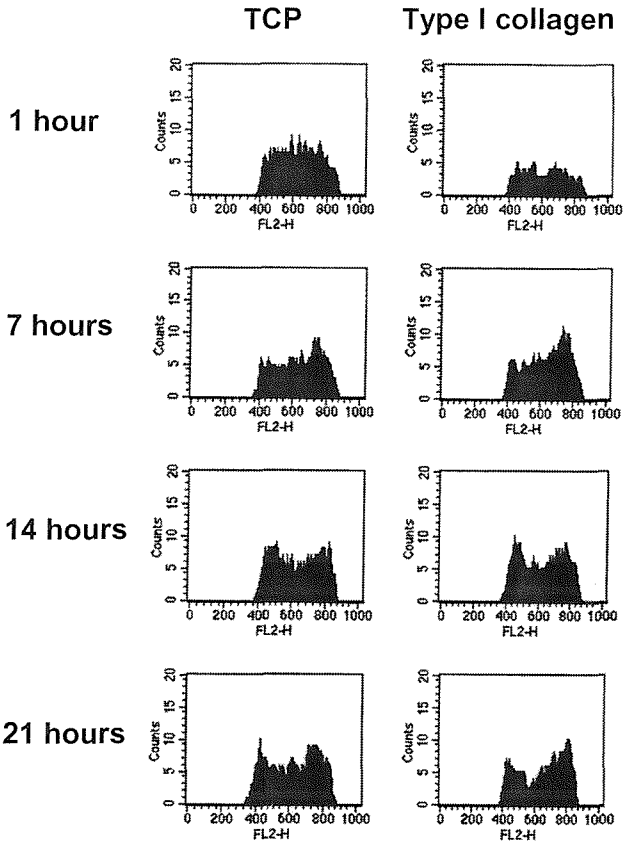


Figure 5.13B
Histograms demonstrating the results of BrdU 'pulse-chase' experiments in AsPC-1 cells
AsPC-1 cells were cultured on TCP or type I collagen using identical experimental protocols to those previously described. Although the pattern is less pronounced, after 14 hours cells can again be identified accumulating ~400 on the x-axis, indicating that cells cultured on type I collagen are accumulating back in G_1 phase earlier than those cultured on TCP (n=1).

This effect was quantified by gating the G_1 peak (see Fig. 5.8) to determine the proportion of cells in G_1 at the latter time points (Fig. 5.14). This indicates that more cells cultured on type I collagen had undergone cell division by 14 hours, compared to TCP. This became less apparent after 21 hours, presumably by which time the some of the cells on type I collagen that were in G_1 had progressed into S phase. Together this evidence indicates that type I collagen increased proliferation of the pancreatic cancer cells by stimulating more rapid cycling. Although the absolute differences were small and not statistically significant, they may have an effect on exponential cell growth in a tumour.

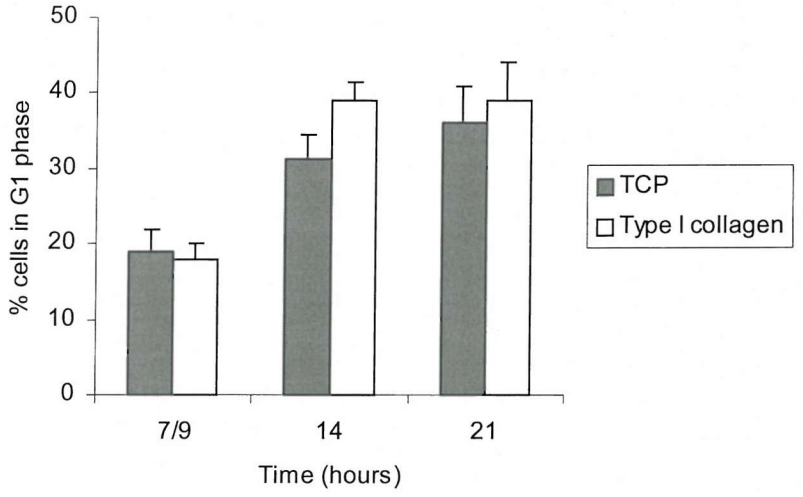


Figure 5.14
Effect of type I collagen on the progress of BrdU labelled Panc-1 cells into G_1 phase
The proportion of BrdU labelled cells in G_1 at the different time-points was quantified by gating histograms generated from Panc-1 cells (n=3, see Fig 5.13A). This demonstrated that with time the proportion of cells in G_1 increases. After 14 hours there were 8% more cells in G_1 on type I collagen (p=0.12) compared to TCP and after 21 hours this difference reduced for reasons discussed in the text. Analysis of data from AsPC-1 cells (n=1, Fig 5.13B) demonstrated a similar pattern, with 4% more AsPC-1 cells cultured on type I collagen in G_1 after 14 hours

3. The role of β_1 integrins in the growth of pancreatic cancer cells

Cells adhere to extracellular matrix predominantly via β_1 integrins (see table in appendix 1) and mediate signals between matrix and cell (Boudreau and Bissell, 1998). β_1 integrin blocking antibodies were therefore employed to determine if the growth regulating effects of type I collagen were β_1 integrin mediated in pancreatic cancer. A pan β_1 integrin blocking antibody (clone 4B4) was initially used in short-term adhesion assays to determine if it blocked adhesion of the pancreatic cancer cells to collagen (Fig 5.15). It caused a dose dependent reduction in adhesion of Panc-1 and AsPC-1 cells. MIA PaCa-2 cells were not used in these experiments because of their relatively inefficient adhesion.

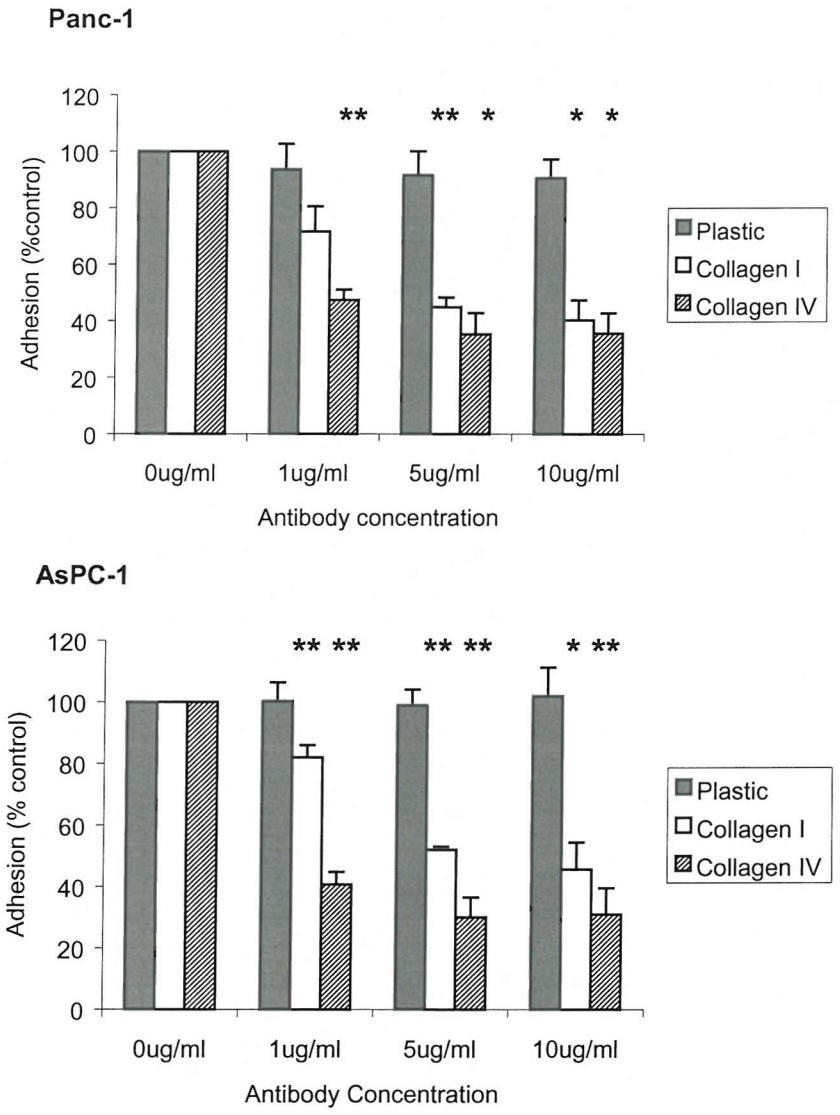


Figure 5.15
Effect of β_1 integrin blockade on adhesion of pancreatic cancer cells to collagen
$5x10^4$ cancer cells that had been incubated with increasing concentrations of β_1 integrin blocking antibody (4B4) were seeded into wells of a 24 well plate prepared with collagen types I and IV or TCP as previously described. Cells were left to attach for 45 minutes, the non-adherent cells were washed off and adherent cells quantified by staining with methylene blue. The antibody did not affect adhesion to plastic but inhibited adhesion to collagen in a concentration dependent manner. Bars represent a mean of 3 independent experiments+/-SEM, *$p<0.05$ and **$p<0.01$. A single repetition of this experiment was undertaken including isotype matched non-immune IgG (as a control), which did not inhibit adhesion.

Having confirmed that the anti β_1 integrin antibody blocked adhesion of pancreatic cancer cells to type I and type IV collagen in short term adhesion assays, it was used at a concentration of 10μg/ml in the tissue culture models and proliferation was measured using ^{3}H-thymidine (Fig. 5.16).

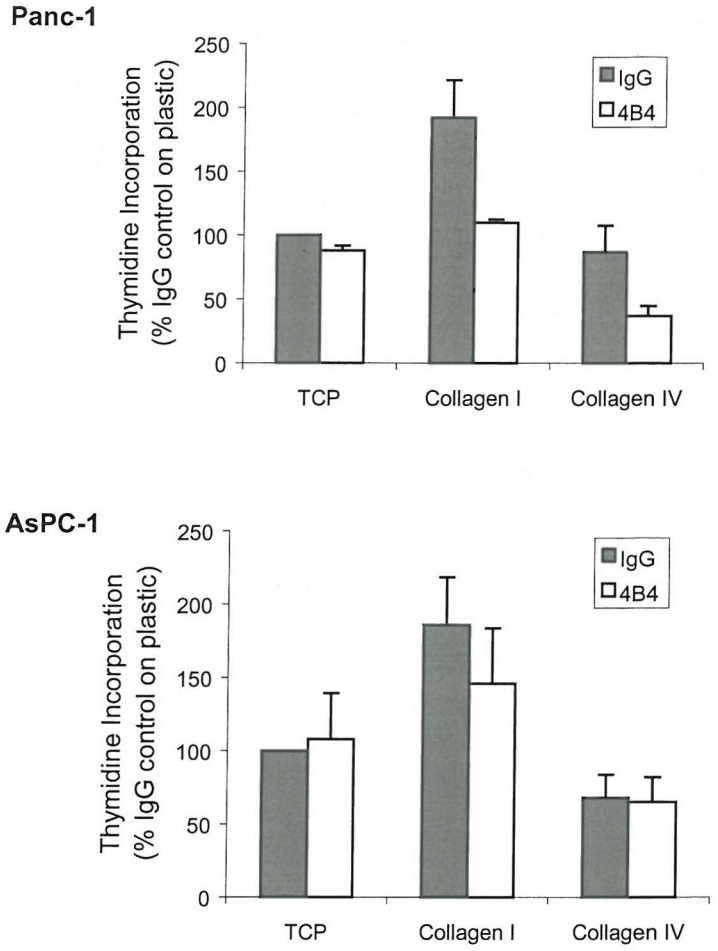


Figure 5.16
Effect of β_1 integrin blockade on ^{3}H-thymidine incorporation by cancer cells cultured on collagen
$1x10^4$ cancer cells were incubated with β_1 integrin blocking antibody 4B4 for 15 minutes prior to seeding onto TCP or collagen type I/IV in normal medium. As with previous experiments, cell were given 24 hours to attach, prior to the medium being replaced with 0.5%FCS and ^{3}H-thymidine subsequently measured. Results were corrected to DNA giving final results in units of cpm/ngDNA. Bars represent the mean of 4 separate experiments +/-SEM and then normalised to those results obtained for cells cultured on TCP with isotype matched non-immune IgG1.

The results in figure 5.16 are presented normalised to the results obtained from cells cultured on TCP in the presence of non-immune IgG providing a graphic image of the overall effect of β_1 integrin blockade. For the purpose of analysis, results were normalised to the isotype non-immune IgG controls on each different matrix (Fig. 5.17).

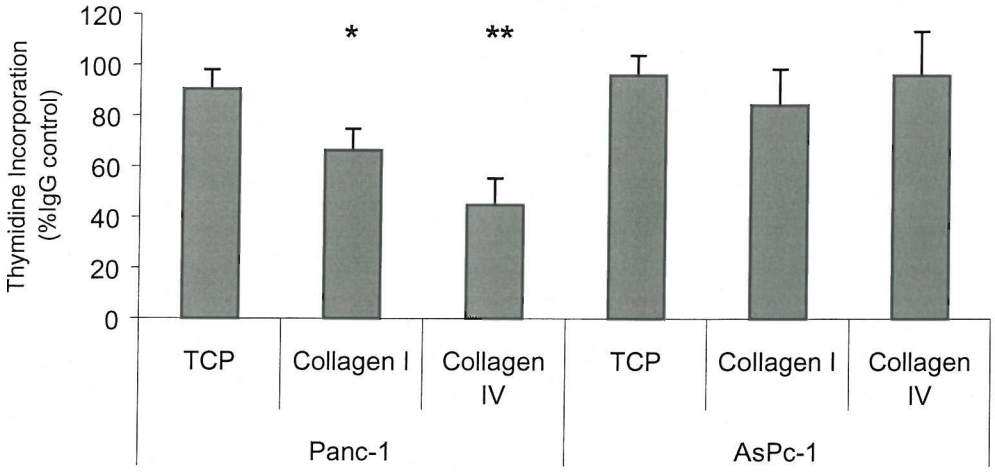


Figure 5.17
Matrix specific effect of β_1 integrin blockade on ^{3}H-thymidine incorporation by cancer cells on collagen
Data presented in figure 5.16 normalised to IgG control on each matrix. Bars represent the mean of 4 separate experiments +/-SEM.

This demonstrated that β_1 integrin blockade was inhibitory to growth of Panc-1 cells on type I collagen and type IV collagen but this effect was not observed in the metastatic AsPC-1 cells. β_1 integrin blockade did not affect ^{3}H-thymidine incorporation on TCP. Together with the results from the adhesion assays this indicates that the pancreatic cancer cells probably adhere to TCP by a β_1 integrin independent mechanism. It was not possible to use these antibodies in the BrdU pulse-chase experiments because of cross-reactivity between mouse derived 4B4 and anti-BrdU antibodies.

4. Investigation of the molecular regulation of pancreatic cancer cell growth

There is good evidence to suggest that integrins signal via Ras pathways, downstream via MAPK/PI3K pathways, ultimately contributing to the regulation of cyclin dependent kinases (CDKs) (Schwartz and Assoian, 2001). Evidence that collagen may regulate expression of cyclin D_1 or the CDK inhibitors p21 and p27 was therefore sought. Protein lysates were made from cells that had been cultured on TCP and types I/IV collagen and subjected to Western analysis as described in Chapter 2 (Fig 5.18).

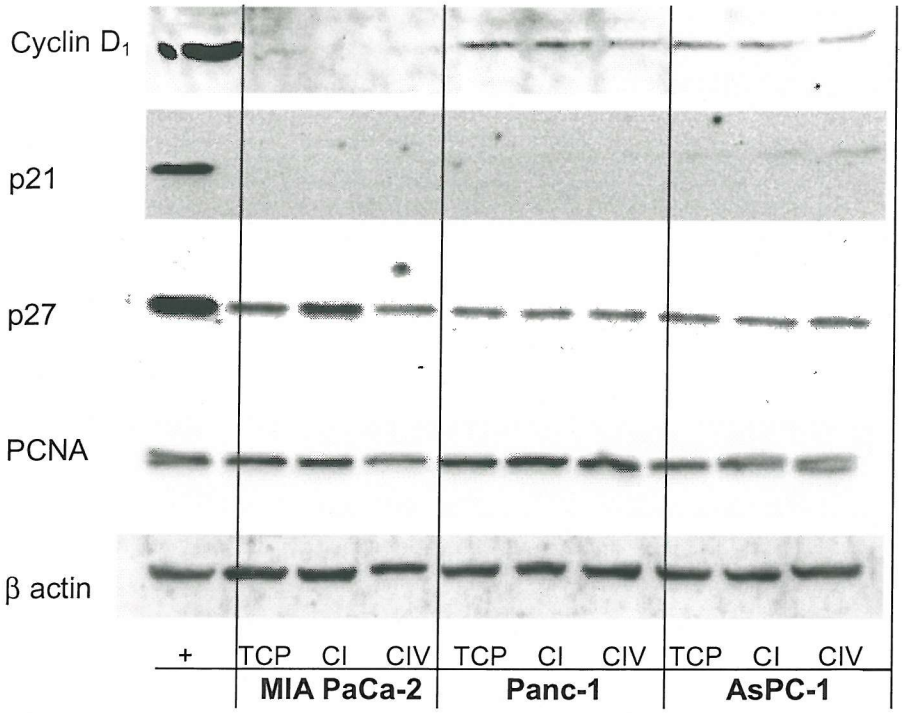


Figure 5.18
Western analysis of cell cycle proteins derived from pancreatic cancer cells cultured on collagen
Pancreatic cancer cells were cultured under conditions identical to those used in the ^{3}H-thymidine assay and harvested at the point ^{3}H-thymidine would have been added. Protein lysates were made (as described in the general methods) and 20μg of protein (60μg for cyclin D_1) subjected to SDS-PAGE prior to identification with antibodies listed in table 2.1. β actin was employed as a loading control and protein lysate from MCF-7 cells (a breast cancer cell line) was used as a positive control (+). PCNA (proliferating cell nuclear antigen, CI (type I collagen), CIV (type IV collagen).

These experiments did not indicate any evidence of regulation of cyclin D_1, p21 or p27 by collagen at this time point in these tissue culture models. PCNA was expressed at a constant level by the cancer cells, irrespective of sub-cellular matrix, which is consistent with repetitive cycling that characterises the phenotype of the pancreatic cancer cell lines *in vitro*.

5. Further investigation of the role of basement membrane in growth of pancreatic cancer cells.

Type IV collagen was relatively inhibitory to the proliferation of pancreatic cancer cells, particularly those derived from primary tumours. Another principal component of basement membrane is laminin, which has also been shown to be relatively inhibitory to growth of pancreatic cancer cells, compared to type I collagen (Mollenhauer et al., 1987). Its effect was therefore tested in the culture models in a purified form and as a component of Matrigel. Matrigel is tumour matrix secreted by

a mouse sarcoma, which also contains type IV collagen and is used as a basement membrane model. Both stimulated ^{3}H-thymidine incorporation by the pancreatic cancer cells relative to TCP and type IV collagen (Fig. 5.19).

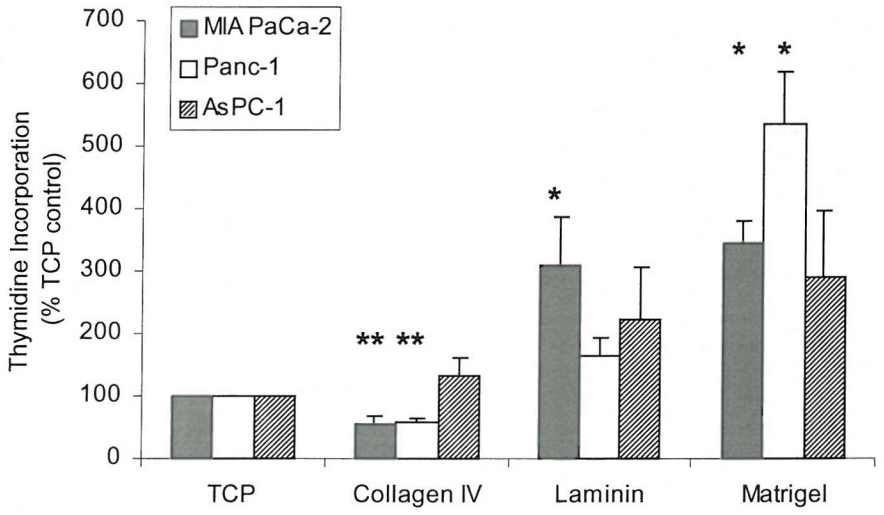


Figure 5.19
Effect of basement membrane components on ^{3}H-thymidine incorporation by pancreatic cancer cells
1x10^{4} pancreatic cancer cells were seeded onto type IV collagen, laminin and matrigel or type IV collagen. After 24 hours the medium was changed to 0.5%FCS and ^{3}H-thymidine incorporation subsequently measured over 16 hours. Results were corrected to DNA giving final results in units of cpm/ngDNA. Bars represent the mean of 3 or more independent experiments +/-SEM, *p<0.05 and **p<0.01, with respect to TCP. The sub-cellular matrices did not alter background scintillation counts. Between independent experiments the range of values in absolute cpm/ngDNA amongst the controls was MIA PaCa-2: 0.54-1.22, Panc-1: 0.53-1.62 and AsPC-1: 1.57-4.23).

In these experiments, laminin stimulated ^{3}H-thymidine incorporation. Matrigel provided a powerful stimulus to ^{3}H-thymidine incorporation but it contains numerous growth factors and the results obtained have to be interpreted in this context.

Basement membrane collagen differs from fibrillar collagen by virtue of retaining non-collagenous domains that facilitate the formation of a mesh-like networks (see Figure 1.3). It has been reported that certain of these non-collagenous domains inhibit a number of cellular processes that may contribute to the malignant phenotype of cancer cells and as such may be potentially therapeutic in oncology (Shahan et al., 1999a; Martinella-Catusse et al., 2001; Shahan et al., 1999b; Pasco et al., 2000b). The peptide sequence responsible for these effects has been identified as a 26 amino acid sequence from the non-collagenous domain of the α3

chain of type IV collagen (α_3(NC1)) (Maeshima et al., 2000). As a derivative of the extracellular matrix with therapeutic potential its effect in these assays was tested. This peptide was synthesised and employed in ^{3}H-thymidine assays to determine if the inhibitory effects of type IV collagen could be reproduced (Fig. 5.20).

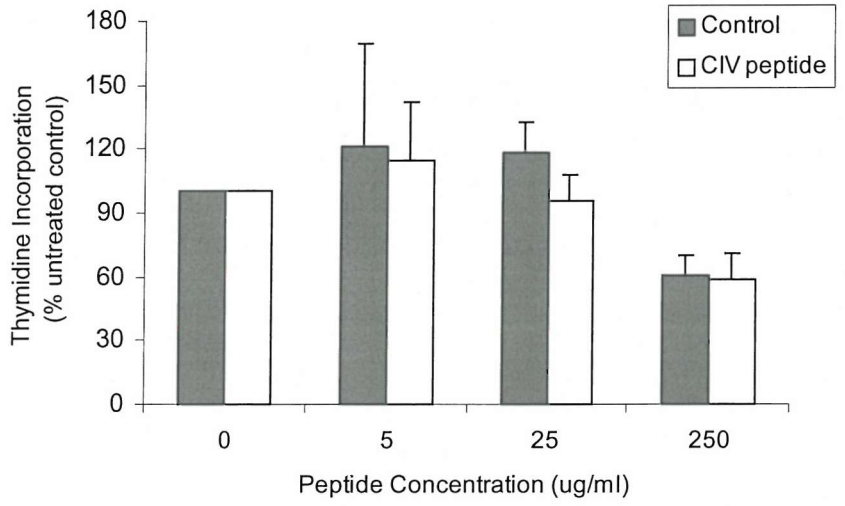


Figure 5.20
Effect of type IV collagen derived peptide on ^{3}H-thymidine incorporation of Panc-1 cells
1x10^4 pancreatic cancer cells were seeded in 24 well plates. After 24 hours the medium was changed to 0.5%FCS v/v DMEM containing α_3(NC1) peptide (or control peptide) was added and^{3}H-thymidine incorporation subsequently measured over 16 hours. Bars represent the mean of 4 independent experiments +/-SEM, *$p<0.05$ with respect to untreated control.

In a series of pilot experiments, both the α_3(NC1) and control peptides induced non-specific inhibition of ^{3}H-thymidine incorporation at high concentrations. There was no evidence to indicate that the α_3(NC1) peptide specifically inhibited proliferation of Panc-1 cells at a concentration of 5μg/ml, which it had previously been shown to do in a variety of tumour cells (that were predominantly mesenchyme derived) (Han et al., 1997).

6. Effect of pancreatic stellate cell derived extracellular matrix on proliferation of pancreatic cancer cells

In view of the varying effects that the extracellular matrices had on cancer cell growth, a series of experiments were performed to determine what the overall effect that PSC derived matrix would have on pancreatic cancer cell growth. The method (described in detail earlier in the chapter) relied firstly on the culture of human PSC until confluent in 24 well plates followed by removal of the cell layer with detergents.

Cancer cells were then seeded onto the PSC derived matrix and their ^{3}H-thymidine incorporation measured. The PSC derived matrix appeared to stimulate ^{3}H-thymidine incorporation in 2 of 3 cell lines (Fig. 5.21). This technique proved difficult to reproduce and the results were not significant but it broadly supported the hypothesis that matrix secreted by PSC would support growth of the pancreatic cancer cell lines *in vivo*.

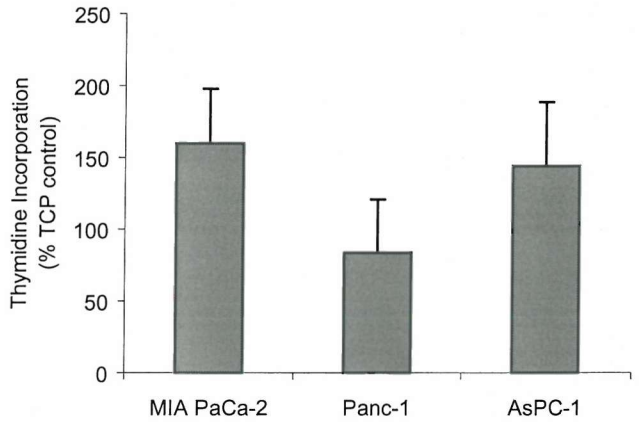


Figure 5.21
Effect of PSC derived matrix on ^{3}H-thymidine incorporation by pancreatic cancer cell lines
1x10^{4} pancreatic cancer cells were seeded onto PSC derived matrix (from 5 separate isolates of PSC). After 24 hours the medium was changed to 0.5%FCS and ^{3}H-thymidine incorporation subsequently measured over 16 hours. Results were corrected to DNA giving final results in units of cpm/ngDNA. Bars represent the mean of 5 independent experiments +/- SEM. ^{3}H-thymidine incorporation was normalised to that measured in duplicate cultures of cancer cells on TCP. Between independent experiments the range of values in absolute cpm/ngDNA amongst the controls was MIA PaCa-2: 1.52-13.7, Panc-1: 5.02-15.26 and AsPC-1: 9.8-21.97).

The main source of variability within the assay was probably related to the deposition of the PSC derived matrix, which was difficult to quantify and therefore to control adequately.

Discussion

Epithelial cells are separated from the interstitial matrix by basement membranes. This anatomical arrangement is of key importance in the diagnosis of epithelial cancer. Dysplastic lesions are morphologically distinct from malignant lesions by virtue of possessing intact basement membrane. Breach of this barrier defines frank malignancy and permits invasion of the malignant cells into the interstitial matrix that contains blood vessels and lymphatics (Hagedorn et al., 2001). The finding that interstitial collagens not only support but actually promote growth of pancreatic cancer cells, relative to basement membrane collagen, is therefore important.

The immunohistochemical studies in Chapter 3 demonstrated that the distribution of type I collagen dramatically increases in comparison to type IV collagen, in the desmoplastic reaction and this finding is supported by other studies (Imamura et al., 1995a; Linder et al., 2001; Yen et al., 2002). These experiments clearly indicate that such a process would favour growth of pancreatic cancer cells and thereby contribute to the malignant phenotype. PSC are likely to promote the change in quality of the matrix in two ways. Firstly, PSC secrete MMP-2 (in excess of pancreatic cancer cells) and this may degrade basement membrane. Secondly, although PSC secrete a multitude of ECM, they principally secrete fibrillar collagen: types I and III, which in purified form supported growth of each pancreatic cancer cell line. Moreover, PSC derived matrix supported growth of pancreatic cancer cells *in vitro*. Study of cell cycle kinetics indicated that type I collagens led to more rapid progression of S phase labelled cells through S phase and G_2M. In context of cells that continually cycle this provided a logical explanation to the experimental findings.

Type IV collagen was present in the desmoplastic reaction but not necessarily in close contact with epithelial cells as is the situation in the normal pancreas. Type IV collagen was inhibitory to growth of the pancreatic cancer cells but much less so to metastatic AsPC-1cells. In itself, this phenotype would promote successful metastasis, where transit through a distant basement membrane is vital, even in the case of AsPC-1 cells that are derived from ascitic metastasis. The general change away from the dominance of type IV collagen distribution in the normal pancreas to the widespread presence of type I collagen in pancreatic cancer is consistent with the deregulated, unstoppable growth of malignant pancreatic cells *in vivo*.

It was hypothesised that the effects of collagen on pancreatic cancer cells were mediated via the previously described integrin signalling pathway that culminates in transcription of cyclin D_1 and ultimately progression into the cell cycle (Schwartz and Assoian, 2001). The possible role of these mechanisms was therefore investigated in these culture models. Classically collagen is ligand for $\alpha_2\beta_1$ and $\alpha_1\beta_1$ integrins and β_1 integrins were therefore blocked to determine their role in the phenotype of the cancer cells cultured on collagen (van der and Sonnenberg, 2001). The β_1 integrin antibodies blocked adhesion of Panc-1 and AsPC-1 to collagen types I and IV in short term adhesion assays. The antibody did not block adhesion to TCP, however, indicating that adhesion to TCP was likely to be β_1 integrin independent. This finding encouraged further investigation of these mechanisms as it appeared that culture on collagen led to integrin ligation whereas culture on TCP did not, thus providing a simple model. The phenotype was however more complex than this and it was not fully deciphered during the course of these experiments.

The effect of β_1 integrin blockade on the proliferation of Panc-1 and AsPC-1 cells yielded some interesting results. It countered the proliferative advantage in Panc-1 cells derived from type I collagen, whilst having no effect on cells cultured on TCP. It also further perturbed the growth of Panc-1 cells on type IV collagen. However, blockade of β_1 integrins in AsPC-1 cells had little effect on cellular proliferation irrespective of matrix. This indicated that other classes of integrin or cell adhesion receptor were also involved, in the AsPC-1 cells at least. Therefore β_1 integrins appeared to play a consistent role in adhesion of the cells to collagen but β_1 integrin blockade did not have a constant effect on growth rates of the cancer cells on different matrices.

Coordinated signalling from receptor tyrosine kinases and integrins stimulates progression from G_1 phase into S phase through induction of cyclin D_1 and repression of p21 and p27 (Assoian and Schwartz, 2001). Type I collagen has been shown to regulate expression of these proteins in prostate cancer and malignant melanoma and they were therefore studied amongst pancreatic cancer cells (Schwartz and Assoian, 2001; Kiefer and Farach-Carson, 2001; Henriet et al., 2000). Cell lysates were made at time points where other experiments had demonstrated differential growth rates on collagen but Western analysis did not indicate that cyclin D_1, p21 or p27 were subject to matrix regulation. The cells were

cultured in serum free media with the aim of maximising integrin signalling over receptor tyrosine kinase signalling but it is possible that any regulation at this level was masked by activating K-ras mutations (harboured by all these cell lines) (Moore et al., 2001). Other reasons as to why this strategy did show any differences included studying the wrong time point or studying an asynchronous signal with a non-kinetic test. Asynchrony could originate from cells adhering to the collagen at different times, which would not lead to a 'flush' of cyclin expression when the cells start to grow. Secondly a protein lysate from a population of cells in perpetual cycle may express a fairly constant level of cyclins and there would not necessarily be a measurable difference in cyclin levels in cells with a reduced time in cycle (cf PCNA). Finally, these proteins may not be regulated at all by matrix in pancreatic cancer.

The study of growth regulation by extracellular matrix is potentially complicated by a number of factors that have been controlled for where possible. Peri-cellular proteolysis is a process whereby proteases expressed by cells lead to matrix remodelling around cells (Liotta and Kohn, 2001; Werb, 1997). This may have 2 consequences. Firstly this may produce proteolytic fragments, which are biologically active and secondly it may release growth factors that are embedded in the matrix (Liotta and Kohn, 2001). Although MMP expression by pancreatic cancer cell lines *in vitro* is generally variable and weak (see Chapter 4), pancreatic cancer cell lines are able to invade through Matrigel (Ellenrieder et al., 2000; Yang et al., 2001). Furthermore, type I collagen has been shown to regulate MMP expression by pancreatic and other malignant cells (Yang et al., 2001; Hornebeck et al., 2002). Pancreatic cancer cells cultured non-degradable on r/r mutant type I collagen were not subject to the same growth stimulus derived from wild type type I collagen. Hence it is expected that production of proteolytic fragments occurred in these tissue culture models. Proteolytic fragments of matrix may promote or inhibit cancer cell growth and migration (Egeblad and Werb, 2002; Ortega and Werb, 2002; Hotary et al., 2003). The importance of growth factors in matrix was demonstrated by culturing pancreatic cancer cells on Matrigel, which is known to be rich in growth factors (Wisdom, Jr. et al., 1992). This was a potential problem with the other matrices but the extraction process for collagen necessitates the use of concentrated acids and alcohols (Glanville et al., 1979). It is possible that this may have denatured non-collagenous proteins but this cannot be confirmed.

In addition to containing growth factors, Matrigel is a gel, which provided the possibility of cellular growth in 3-dimensions, although cells were plated onto a layer of Matigel, rather than suspended in it. Epithelial cells normally grow in 2-dimensions on basement membrane. Therefore invasion into interstitial matrix not only exposes cells to different collagen but also growth in 3-dimensions (which requires peri-cellular proteolysis)(Hotary et al., 2003). There is a growing body of evidence that indicates cells can behave differently when cultured in gels (this is best illustrated in studies of breast cancer and is discussed further in Chapter 8) (Abbott, 2003; Bissell et al., 2002). It is increasingly recognised that tensional forces on cells mediated by cell binding to the extracellular matrix, associated with changes in cell shape and cytoskeletal tension all contribute to the 'spatial' control of cell-cycle progression (Huang and Ingber, 1999). Three dimensional culture systems are difficult to work with and investigation in this field remains in its infancy but it is likely to represent the future of this type of research (Abbott, 2003). Of course it is not just 3-dimensional matrix that is important, growth of cells in 3-dimensions can also have a profound effect on phenotype (Yamada and Clark, 2002).

Conclusions

Collagen types I and III promote growth of pancreatic cancer cells relative to TCP and type IV collagen but the differential effect of this is reduced in metastatic cells. This is caused by type I collagen reducing the time taken for cells to progress through the cell cycle. β_1 integrins may in part mediate this effect but the exact mechanisms remain elusive.

Chapter 6

Role of extracellular matrix in regulating pancreatic cancer cell chemosensitivity

Introduction

With the observation that fibrillar collagen supported of growth pancreatic cancer cells, it was logical to investigate whether collagen also reduced apoptosis. Chemotherapy typically induces apoptosis in cancer cells and it was decided to address this hypothesis by treating the pancreatic cancer cells with 5-fuorouracil (5-FU), an agent that is used to treat patients suffering with pancreatic cancer.

Hypothesis

Cancer cells derive a survival advantage from collagen through increased resistance to apoptosis.

Aims

1. To determine whether matrix regulates cancer cell apoptosis.
2. To determine if collagen provides cancer cells with a survival advantage.

Methods

The effect that collagen had on apoptosis and long-term survival of pancreatic cancer cells treated with 5-FU (F.H. Faulding & Co.) was studied with a number of techniques. Cells ($8x10^5$) were cultured in $25cm^2$ flasks prepared with collagen as described in Chapter 2 (where indicated). After 24 hours in normal medium, it was changed to 0.5%FCS v/v DMEM containing 5-FU (2-500μg/ml) and the cells were incubated for a further 24-96 hours. The cells were then counted, subjected to FACS or lysates made for Western analysis or caspase assay. Cells were also removed from the chemotherapy and their clonogenic potential subsequently measured. The varying concentrations of 5-FU used reflected the varying requirement for apoptotic synchrony by different assays.

1. Determination of apoptosis by cellular morphology

A defined set of morphological changes can be identified in cells undergoing apoptosis: nuclear condensation and fragmentation and membrane blebbing (Martin and Lenardo, 1998). Such changes in nuclear morphology were identified by staining cells (in situ) with acridine orange (Sigma, final concentration 1μg/ml) (Martin and Lenardo, 1998). Membrane blebbing was observed with light microscopy. Dose response experiments (to 5-FU) were undertaken using this method. $1x10^4$ cancer cells were seeded into wells of a 24 well plate and cultured in normal medium for 24 hours. The medium was then changed to 0.5%FCS v/v DMEM containing increasing concentrations of 5-FU. The cells were then incubated for up to 72 hours prior to the fraction of apoptotic bodies being counted. This was done by randomly selecting 3 high power fields and counting the number of cells with normal nuclear morphology and apoptotic nuclear morphology (including those detached in the media) in triplicate wells. The apoptotic fraction was calculated by dividing the apoptotic fraction by the total number of cells counted.

2. Caspase activity assay

Caspase-3 is an effector caspase expressed by cells committed to apoptosis and its expression was studied using a commercial Caspase-3 Cellular Activity Assay Kit (Calbiochem), using the manufacturers instructions. The cells were collected from the culture model described above (including cells suspended in the culture media) and lysed in the kit lysis buffer. The protein concentration in the lysates was measured (method Chapter 2) and 30μg protein entered into the caspase-3 assay. The assay is based on cleavage of a DEVD peptide substrate by caspase-3, releasing p-nitroaniline that resulted in a colour change. The change in optical density (at 405nm), was measured over a period of up to 10 hours and plotted against time. The slope directly correlated to caspase-3 activity and caspase activity was calculated from the following equation:

$$\text{Caspase activity (pmol/min/μg protein)} = \frac{\text{Slope } (\Delta A/\text{min}) \times \text{assay volume}}{\mu g \text{ of protein}}$$

The assay volume (100μL) and protein used in the assay (30μg) were constant.

3. Quantifying apoptosis by counting

Cells were cultured as described and apoptosis quantified using a method described by Diaz et al (Diaz et al., 2000). After treatment with 5-FU the detached fraction of cells (suspended in the media) was collected separately from the detached cells, which were trypsinised. After centrifugation the cells were suspended in equal volumes of PBS and counted (in triplicate) using a $1mm^3$ haemocytometer. The nuclear morphology (acridine orange) and PARP expression (Western analysis) of the adherent and detached fractions were also studied.

4. Clonogenic Assays

The clonogenic assay can be used to quantify cell survival following an injurious stimulus, such as treatment with chemotherapy. It seeks to detect how many cells remain able to form colonies (by undergoing cell division) after injury and as such provides powerful evidence of the effect of agents on the long-term survival of cells. Cells were cultured in $25cm^2$ flasks (as described above) on TCP or collagen types I or IV and exposed to 5-FU for 24 hours (with a paired untreated control). After this the 5-FU was removed, the cells were trypsinised and 200 cells seeded directly onto untreated TCP in wells of a 24 well plate. These were returned to the incubator and left for 8-14 days until the cells had formed colonies. Media was routinely changed every 7 days. The wells were then washed with PBS and the colonies were fixed with absolute methanol. Once dry they were stained with undiluted Giemsa stain (Sigma) for 2 minutes before being washed in dH_2O and air dried (Fig 6.1).

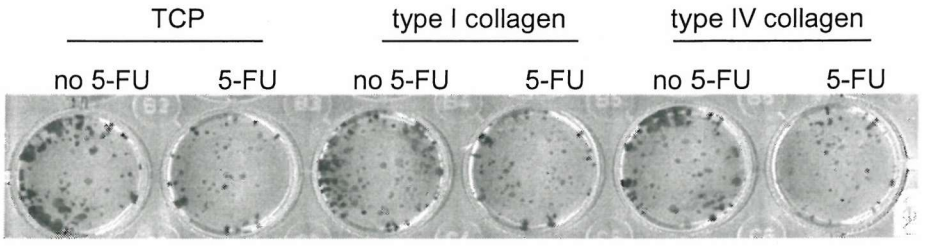


Figure 6.1
Example of Clonogenic Assay
The appearance of MIA PaCa-2 cells subjected to clonogenic assay on TCP and collagen types I and IV after treatment with 3μg/ml5-FU (stained with Geimsa stain).

The colonies were counted using a dissecting microscope with the culture plate placed on a printed grid. This grid contained a series of dots, which corresponded to the size of a colony comprising 16 cells, in order to make the counting protocol

structured and reproducible. From each plate, a clonogenic index was calculated using the following formula:

$$\text{Clonogenic Index} = \frac{\text{Colony number in treated wells}}{\text{Colony number in untreated wells}}$$

The mean clonogenic index from quadruplicate plates was then entered into inter-experimental analysis.

Results

1. 5-fluorouracil induces apoptosis in pancreatic cancer cells

Three lines of evidence indicated that 5-FU induced apoptosis in the pancreatic cancer cell lines. Firstly the morphological hallmarks of apoptosis were displayed by cells treated with 5-FU, with evidence of nuclear condensation/fragmentation and membrane blebbing (Fig. 6.2A and B). Secondly there was evidence of caspase-3 activation in pancreatic cancer cells cultured with 5-FU (Fig. 6.2C). Caspases are the enzymes responsible for the process of cellular execution, which once activated is irreversible. PARP is a cellular DNA repair enzyme that is cleaved by caspases during apoptosis producing a characteristic 85kDa fragment identifiable by Western analysis. Cleaved PARP was present in cells treated with 5-FU (Fig. 6.2D).

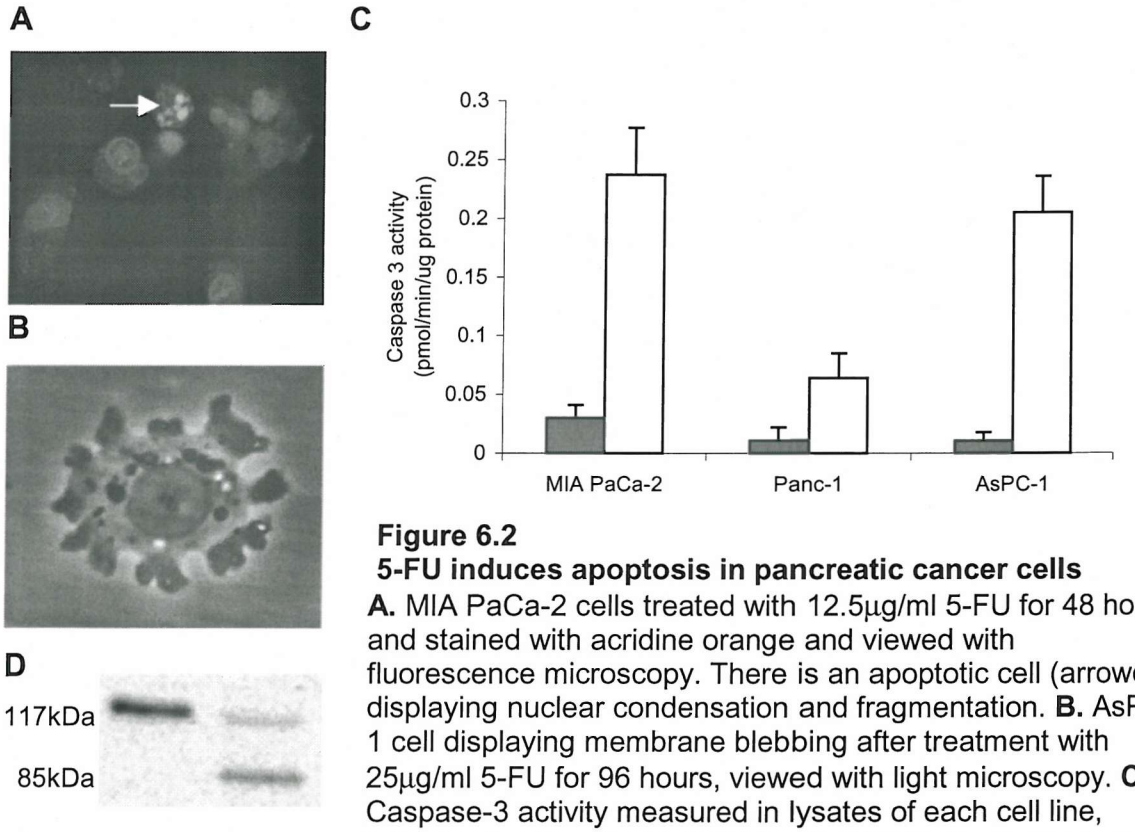


Figure 6.2
5-FU induces apoptosis in pancreatic cancer cells
A. MIA PaCa-2 cells treated with 12.5μg/ml 5-FU for 48 hours and stained with acridine orange and viewed with fluorescence microscopy. There is an apoptotic cell (arrowed) displaying nuclear condensation and fragmentation. **B.** AsPC-1 cell displaying membrane blebbing after treatment with 25μg/ml 5-FU for 96 hours, viewed with light microscopy. **C.** Caspase-3 activity measured in lysates of each cell line, following treatment with 5-FU (n=4) (MIA PaCa-2: 12.5μg/ml 5-FU for 24 hours, Panc-1/AsPC-1: 500μg/ml 5-FU for 72 hours). **D.** Western analysis of protein lystates made from untreated AsPC-1 cells (left) and AsPC-1 cells treated with 25μg/ml 5-FU for 96 hours (right). Native PARP has a molecular weight of 117kDa and PARP cleaved during apoptosis 85kDa.

Having identified that 5-FU induced apoptosis in each of the pancreatic cancer cell lines, a series of experiments were undertaken to determine the dose response curves for each cell line (Fig. 6.3). MIA PaCa-2 cells were relatively chemosensitive but to induce apoptosis in Panc-1 and AsPC-1 cells, higher does of 5-FU were required.

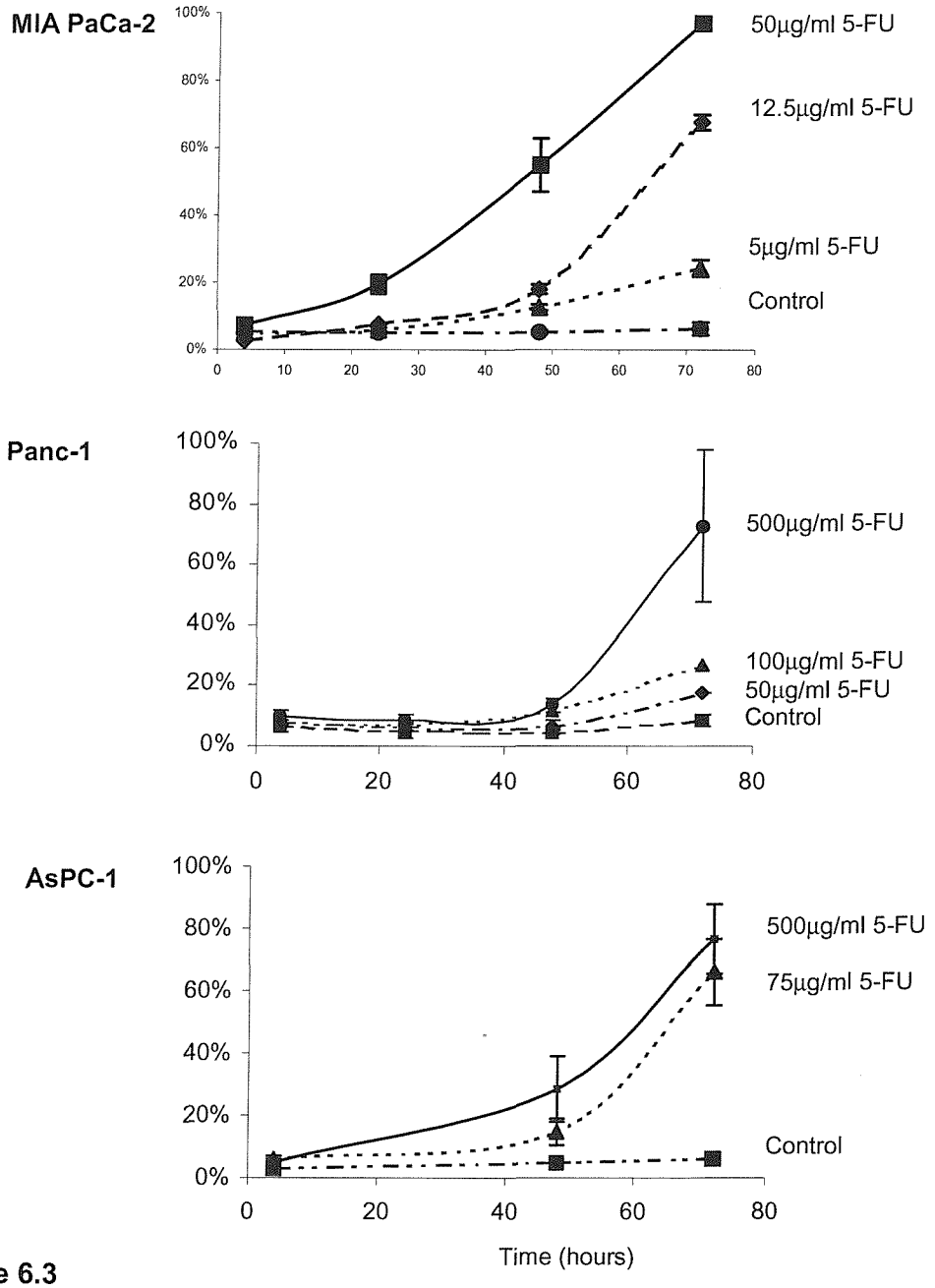


Figure 6.3
Dose response curves of pancreatic cancer cells to 5-FU
The apoptotic fractions (y-axis) were determined by counting the number of apoptotic cells (stained with acridine orange) in cultures of pancreatic cancer cells treated with 5-FU. The number of apoptotic cells was divided by the total number of cell (apoptotic and non-apoptotic) in high power fields, to determine the apoptotic fraction. The data points represent the mean of between 2 and 6 independent experiments +/-SEM.

Having identified 5-FU as a clinically relevant agent that induced apoptosis, it was used in experiments to determine if collagen also regulated chemosensitivity amongst the pancreatic cancer cells. Pilot experiments conducted in parallel with the dose response experiments (described above, Fig 6.3), indicated that at high doses of 5-FU the rate of apoptosis was the same between cells cultured on plastic and collagen. Apoptosis was therefore studied in models where cells were exposed to lower doses of 5-FU for longer time periods. In doing so, apoptosis did not occur as a synchronous event over a short period but rather at a continuous low level. Western analysis of PARP was found to be a good method of quantifying apoptosis in these culture models. PARP cleavage products are not degraded and therefore accumulated within the tissue culture model. A series of screening experiments was then undertaken to study the effect of type I collagen and type IV collagen, compared to TCP. 5-FU induced PARP cleavage in all 3 cell lines in a time and dose dependent manner. This was exemplified by the results obtained using Panc-1 cells where no PARP cleavage was evident at 48 hours but after 96 hours, cleaved PARP was expressed by cells treated with chemotherapy (Fig 6.4).

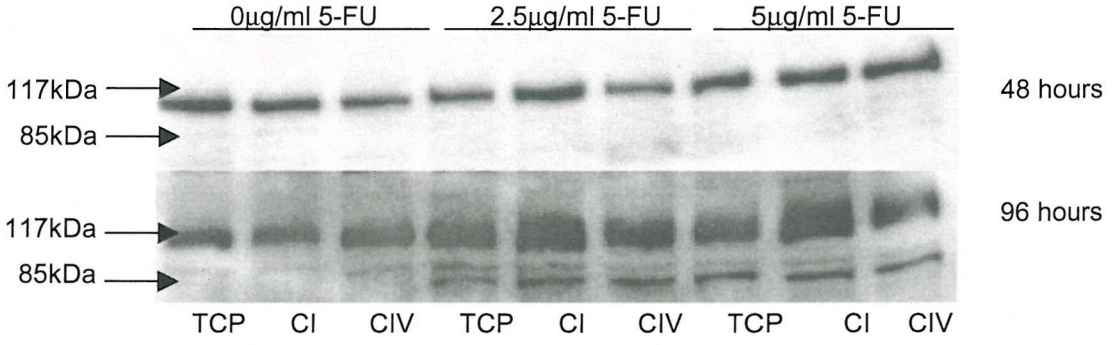


Fig 6.4
Western blots demonstrating the effect of collagen on PARP cleavage in Panc-1 cells treated with 5-FU
Panc-1 cells were cultured on TCP and collagen types I and IV as previously described. After 24 hours the normal culture medium containing 10% FCS was removed and replaced with 0.5%FCS v/v DMEM containing increasing concentrations of 5-FU. Protein lysates were made at the time points indicated and equal quantities of protein separated by SDS-PAGE. Native PARP has a molecular weight of 117kDa and cleaved PARP 85kDa. This image (obtained by autoradiography) was representative of 2 independent experiments.

Despite the differential growth effects that collagen exerted over Panc-1 cells, there was no evidence that it regulated apoptosis in the same way. A similar pattern of PARP cleavage was observed in MIA PaCa-2 cells (Fig 6.5). There was dose dependent PARP cleavage but this was not regulated by matrix. There was also evidence of PARP cleavage in the untreated controls, which reflects the relatively

high basal rate of apoptosis in cultures of MIA PaCa-2. This was reflected by relatively high caspase-3 activity in the untreated controls (see Fig. 6.1).

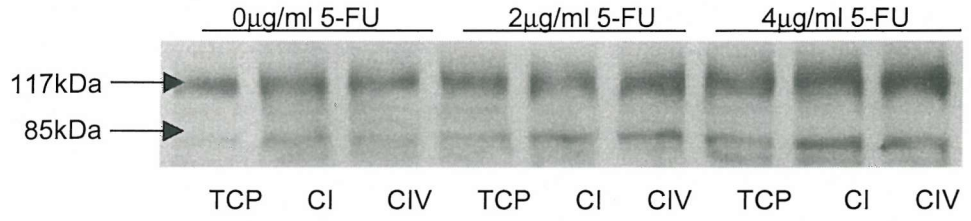


Fig 6.5
Western blot demonstrating the effect of collagen on PARP cleavage in MIA PaCa-2 cells treated with 5-FU
MIA PaCa-2 cells were cultured on TCP and collagen types I and IV as previously described. After 24 hours the normal culture medium containing 10% FCS was removed and replaced with 0.5%FCS v/v DMEM containing increasing concentrations of 5-FU. Protein lysates were made after 72 hours and equal quantities of protein separated by SDS-PAGE. This image (obtained using autoradiography) was representative of 2 independent experiments.

When these experiments were repeated using AsPC-1 cells, PARP cleavage increased with the time and dose of 5-FU (Fig 6.6). However, there was evidence of a reduction in PARP cleavage in cells cultured on type I collagen compared to type IV collagen and TCP. This was only seen in cells treated with 25μg/ml 5-FU. At higher doses only cleaved PARP was identified, indicating widespread apoptosis, irrespective of the sub-cellular matrix.

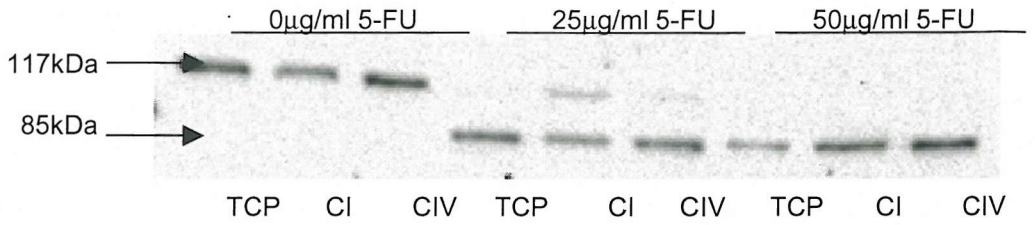


Fig 6.6
Western blot demonstrating the effect of collagen on PARP cleavage in AsPC-1 cells treated with 5-FU
AsPC-1 cells were cultured on TCP and collagen types I and IV as previously described. After 24 hours the normal culture medium containing 10% FCS was removed and replaced with 0.5%FCS v/v DMEM containing increasing concentrations of 5-FU. Protein lysates were made after 96 hours and equal quantities of protein separated by SDS-PAGE. This image is representative of 3 independent experiments.

These experiments indicated that although type I collagen consistently increased proliferation of all 3 pancreatic cancer cell lines, it had no consistent effect on the regulation of apoptosis. The finding that AsPC-1 cells were subject to regulation was nonetheless an important observation. *In vivo* tumours contain numerous clones which may display various phenotypes (this may explain why some cells metastasise, whilst others may be resistant to chemotherapy etc.). The effect that type I collagen had on apoptosis of AsPC-1 cells was therefore studied further because the above observation suggested that the regulation of apoptosis may have also been important mechanism regulating cancer cell growth.

2. The effect of type I collagen on apoptosis of AsPC-1 cells

When examining cultures of AsPC-1 cells treated with 5-FU, there was an obvious difference between those cultured on type I collagen and TCP. Light microscopy indicated a marked reduction in the number of AsPC-1 cells growing on TCP compared to cells cultured on type I collagen, after 72 hours treatment with 5-FU. This was quantified by counting the adherent and detached cells (Fig. 6.7).

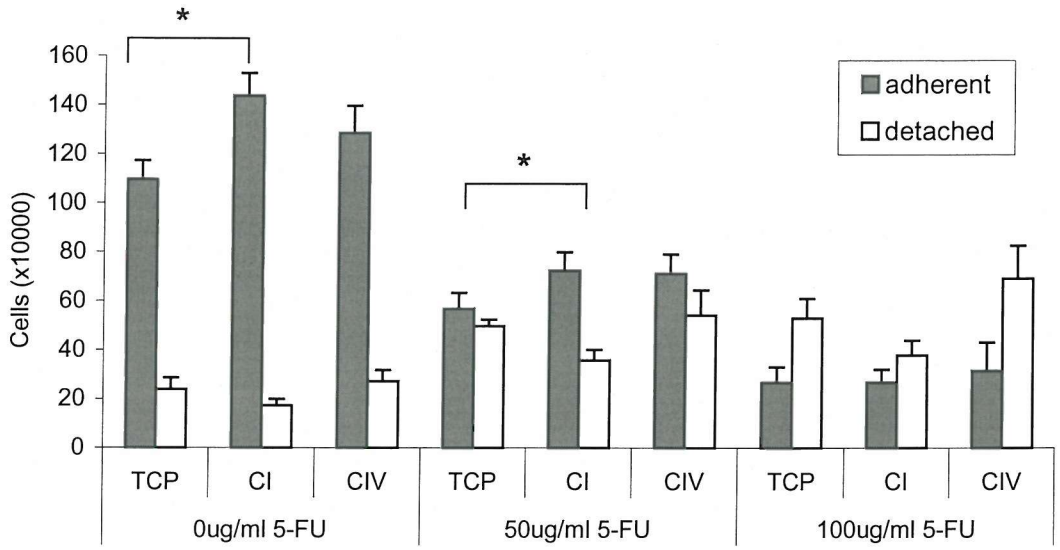


Figure 6.7
Quantification of the effect of type I collagen on 5-FU treated AsPC-1 cell viability
AsPC-1 cells were treated with 5-FU for 72 hours. The detached cells were harvested from the medium, whilst the adherent cells were harvested by trypsinisation. Both fractions were suspended in equal volumes PBS and counted using a haemocytometer. The bars represent a mean of 5 independent experiments +/-SEM
(* $p<0.05$ with respect to relevant control on TCP).

These experiments confirmed the pro-proliferative effect of type I collagen, with the total number of cells greater on type I collagen than TCP. This held true for the number of cells in cultures treated with lower doses of 5-FU but not at the higher doses of 5-FU. There was also a relative reduction in the number of detached cells amongst AsPC-1 cells cultured on type I collagen. It was suspected that the detached cells were apoptotic and this was confirmed in 3 ways (Fig. 6.8). The adherent cells had normal nuclear morphology (determined by acridine orange staining), whilst the detached cells had apoptotic morphology, with nuclear condensation and fragmentation (Fig. 6.8A and B). Furthermore, when PARP expression was analysed, there was a clear division of native PARP expression by the adherent cells and cleaved PARP amongst the detached cells (Fig. 6.8C). Non-viability of the detached cells was ensured by removing them from the 5-FU and returning them to normal culture medium. After 10 days there was no evidence of cells becoming adherent or forming colonies.

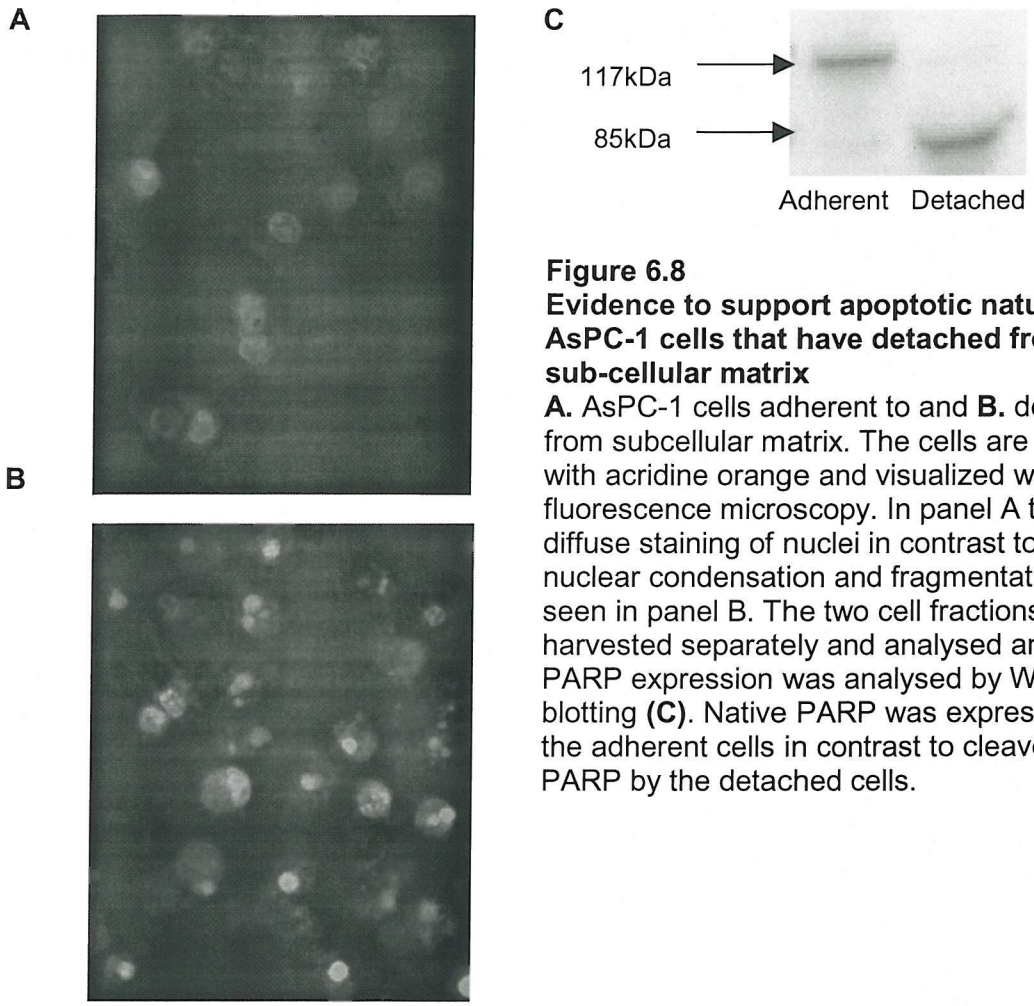
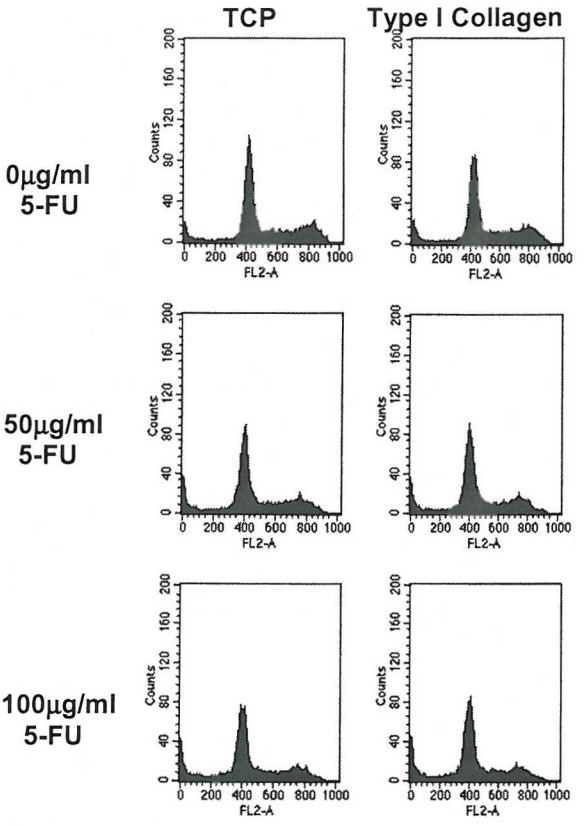


Figure 6.8
Evidence to support apoptotic nature of AsPC-1 cells that have detached from sub-cellular matrix
A. AsPC-1 cells adherent to and **B.** detached from subcellular matrix. The cells are stained with acridine orange and visualized with fluorescence microscopy. In panel A there is diffuse staining of nuclei in contrast to nuclear condensation and fragmentation seen in panel B. The two cell fractions were harvested separately and analysed and PARP expression was analysed by Western blotting **(C)**. Native PARP was expressed by the adherent cells in contrast to cleaved PARP by the detached cells.

The regulation of AsPC-1 cell apoptosis was also studied by FACS analysis after staining cells with propidium iodide (Fig. 6.9). As the concentration of 5-FU was increased, the proportion of cells in the sub-G_0 area increased. Examination of the histograms did not indicate that the proportion of sub-G_0 (and therefore apoptotic cells) was altered in cells cultured on TCP and type I collagen. This was confirmed by quantifying cells in the sub-G_0, G_1, and S/G_2M phases, by gating the individual populations (described earlier, Fig 5.8) (Fig. 6.10).

Figure 6.9
Effect of type I collagen on sensitivity of AsPC-1 cells to 5-FU measured by FACS
AsPC-1 cells were cultured on TCP or type I collagen for 24 hours in normal cell culture medium prior to treatment with increasing concentrations of 5-FU in 0.5%FCS v/v DMEM. After 72 hours the cells were harvested, including those floating in the medium. These were stained with PI and analysed with FACS. The histograms shown are representative of 4 separate experiments.



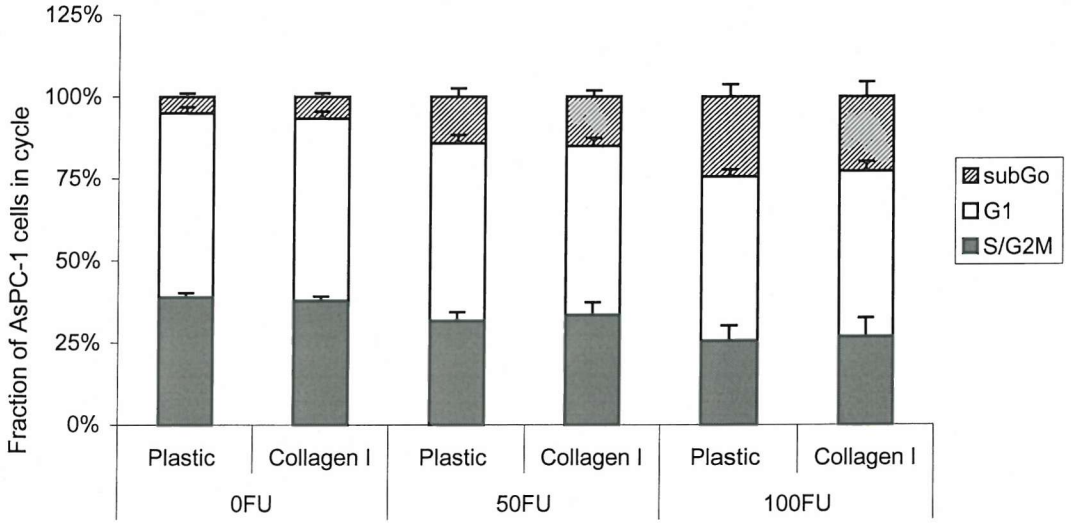


Figure 6.10
Quantification of the effect of type I collagen on apoptosis of AsPC-1 cells exposed to 5-FU by FACS analysis
This chart was derived from gating histograms generated by staining AsPC-1 cells with PI after culture with 5-FU (as described in figure 6.7). The mean (+/-SEM) of data from 4 separate experiments is shown. There were no significant statistical differences.

The figure demonstrates that the number of apoptotic cells comprising the sub-G_0 fraction increases with increasing dose of 5-FU but does not identify a significant difference in this fraction in cells cultured on type I collagen compared to cells cultured on TCP. As the fraction of apoptotic cells increases, the proportion of cells in S and G_2M phases reduces, whilst the fraction of cells in G_1 remains relatively constant. This indicates that 5-FU also induces relative growth arrest but this was not altered by type I collagen either.

Hence, together these experiments confirmed that type I collagen led to a modest but significant reduction in apoptosis of AsPC-1 cells cultured on type I collagen compared to TCP. To a lesser extent this was true of type IV collagen. The apparent discrepancy in the results from the FACS analysis is likely to be due to a combination of changes in cell number (resulting from the proliferative advantage derived from type I collagen) and a failure of oligonucleosomal degradation during apoptosis, both of which are considered fully in the discussion.

3. Molecular regulation of apoptosis in AsPC-1 cells

In order to determine the mechanism by which type I collagen conferred chemoresistance to AsPC-1 cells treated with 5-FU, expression of the Bcl-2 protein family was studied. Protein lysates were made from cell cultures in which the changes in apoptosis had been observed and subjected to Western analysis (Fig. 6.11).

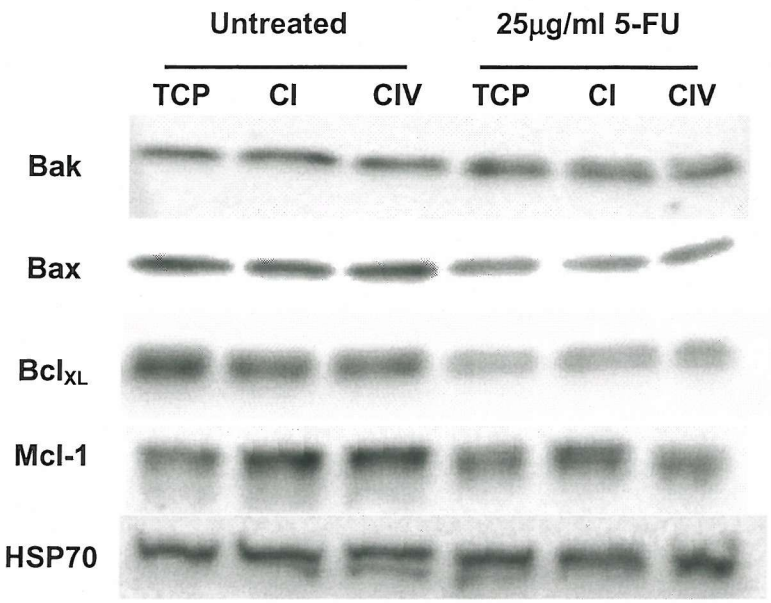


Figure 6.11
Western analysis of apoptotic regulators in AsPC-1 cells treated with 5-FU
AsPC-1 cells were cultured on either TCP or collagen types I/IV for 24 hours before the medium was replaced with 0.5%FCS v/v DMEM (untreated) or 0.5%FCS v/v DMEM containing 25μg/ml 5-FU. After 72 hours protein lysates were made and equal quantities subjected to SDS-PAGE. Members of the Bcl-2 family/heat shock protein 70 (HSP 70) were identified with the appropriate primary/secondary antibody complexes (see table 2.1) and visualised using a HRP sensitive chemiluminescent substrate. These showed that amongst the cells treated with 5-FU, mcl-1 was subject to regulation by type I collagen and may therefore contribute to the regulation of apoptosis by type I collagen. The images are representative of 2 or more independent experiments, with the exception of HSP 70, which was obtained re-probing another membrane on a single occasion.

There were a number of changes in expression of bcl-2 family members between AsPC-1 cells treated with 5-FU and the untreated controls that were consistent with apoptosis induced by 5-FU. The principal changes in the pro-apoptotic proteins was marked up-regulation of Bak expression and modest down-regulation of Bax. Of the anti-apoptotic proteins Bcl_{XL} expression was markedly down regulated on treatment with 5-FU and this was accompanied by a modest reduction in mcl-1. Mcl-1 was expressed at higher levels in cells cultured on collagen types I and IV, compared to TCP. This level of expression was maintained in 5-FU treated cells cultured on type I collagen, compared to TCP and type IV collagen. This may underlie the anti-apoptotic effect that type I collagen has on AsPC-1 cells. Although not a member of the bcl-2 family, Western blots of HSP70 are included in figure 6.11 because it a cytoplasmic protein with known cyto-protective qualities. There was no evidence of that it was induced by treating the cells with 5-FU or that it was regulated by

collagen. It was therefore not considered to play a role in apoptosis in these culture models.

In summary, 5-FU induces apoptosis amongst pancreatic cancer cells. Type I collagen modestly reduced apoptosis of AsPC-1 cells but not apoptosis of MIA PaCa-2 or Panc-1 cells. Experimental evidence suggests that one potential mechanism underlying this phenomenon was the maintenance of relatively high mcl-1 expression in AsPC-1 cells cultured on type I collagen, compared to TCP and type IV collagen.

4. Role of type I collagen in the long-term survival of pancreatic cancer cells.

Having identified that type I collagen played an important role in the growth and chemoresistance of pancreatic cancer cells, its effect on the long-term survival of the cancer cells was studied using clonogenic assays. The long-term survival of cancer cells is clinically relevant because it is these cells that repopulate tumours after cycles radio or chemotherapy (Steel, 2001). In these experiments, the pancreatic cancer cell lines were exposed to low dose 5-FU, whilst cultured on TCP, type I collagen and type IV collagen. Their colony forming ability was subsequently assessed and compared to untreated controls (Fig 6.12). This method gave an index of cell viability, because only viable cells were able to divide and thus form colonies. Two hundred cells were seeded into each well and the colony forming efficiency was 13.3-22.9% amongst the untreated controls (i.e. there were a mean of 26.6-45.8 colonies in the control wells). Culturing the cells on collagen did not significantly change this colony forming efficiency amongst the untreated control cells (table 6.1) and was thus excluded a non-specific effect that may have indirectly altered colony numbers amongst the treated cells. Having established this, it was possible to quantify the specific effect that collagen had on clonogenic index (colony number in treated wells/colony number in untreated wells) of cells treated with 5-FU (Fig. 6.12). This repeatedly demonstrated that type I collagen provided the cancer cells with a clear long-term survival advantage in the face of treatment with 5-FU. Type IV collagen had a more variable effect, in which the cell lines derived from primary tumours (MIA PaCa-2 and Panc-1) derived some protection from type IV collagen (significantly so in Panc-1 cells) but not the metastatic AsPC-1 cells.

	Plastic		Type I collagen		Type IV collagen	
	Untreated	Treated	Untreated	Treated	Untreated	Treated
MIA PaCa-2	100+/-0	32.0+/-7.6	111.0+/-15.6	45.0+/-9.6	105.4+/-15.6	46.9+/-9.6
Panc-1	100+/-0	21.1+/-4.1	95.8+/-9.7	32.9+/-9.6	95.3+/-11.7	30.7+/-6.8
AsPC-1	100+/-0	28.7+/-1.5	88.7+/-4.5	37.5+/-12.1	98.4+/-5.8	25.8+/-9.5

Table 6.1
Effect of collagen on background colony forming efficiency of pancreatic cancer cells lines
Collagen led to minor background variations in colony forming efficiency of pancreatic cancer cells in the untreated wells. The results are normalized to mean results obtained on TCP, which was given an arbitrary value of 100 (+/-SEM) from 4 or more independent experiments.

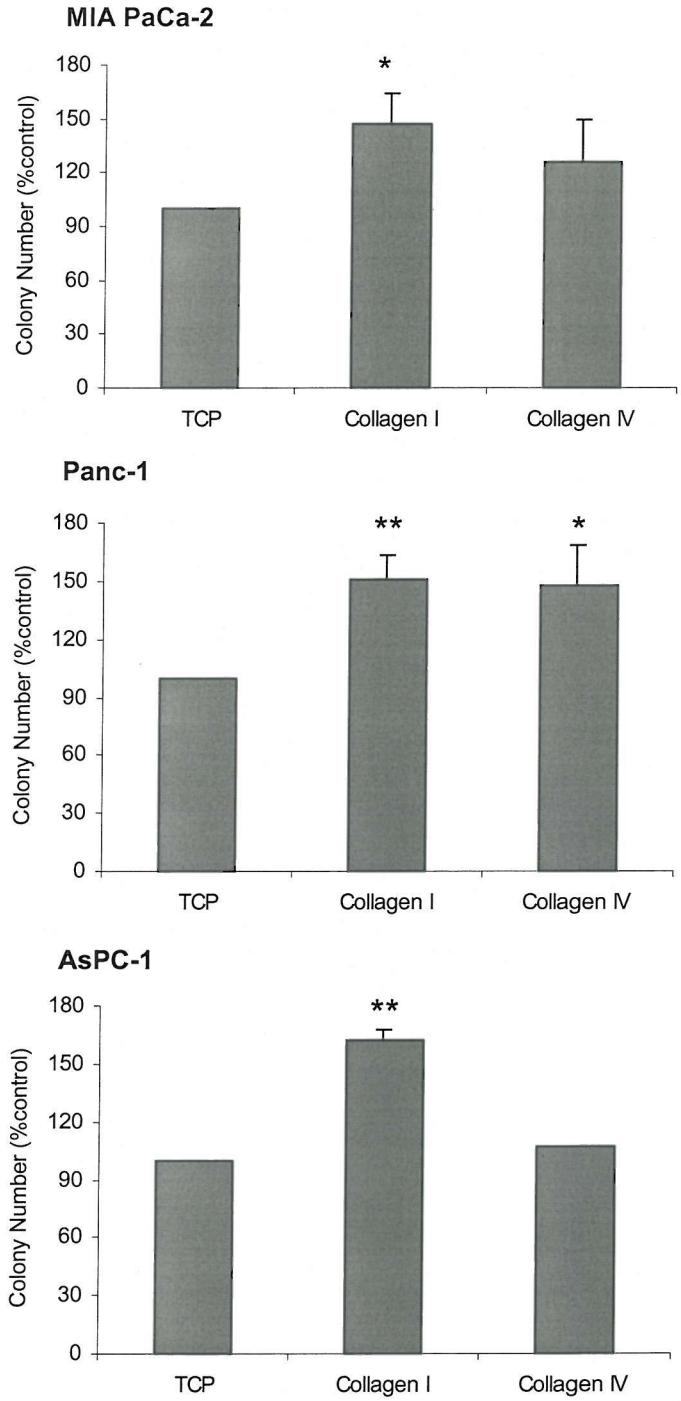


Figure 6.12
Effect of collagen on clonogenic potential of pancreatic cancer cells treated with 5-FU after correction for background variation of colony number
The pancreatic cancer cells were cultured on either TCP or collagen types I/IV for 24 hours before the medium was replaced with 0.5%FCS v/v DMEM (untreated) or 0.5%FCS v/v DMEM containing 5-FU (MIA PaCa-2: 3μg/ml, Panc-1: 7μg/ml, AsPC-1: 5μg/ml). After 24 hours adherent cells were harvested using trypsin and 200 cells seeded into 24 well plates, in normal media. The plates were left for 8-14 days, until colonies had formed. Colonies of >16 cells were counted and the mean clonogenic index expressed as a percentage of TCP controls, which were given an arbitrary value of 100%. Bars represent the mean of 4 or more independent experiments +/- SEM (*$p<0.05$, **$p<0.01$ with respect to TCP).

Discussion

In contrast to the consistent stimulus to proliferation that all 3 pancreatic cancer cell lines derived from type I collagen, it did not consistently regulate apoptosis. Type I collagen significantly reduced apoptosis of AsPC-1 cells compared to TCP and a lesser extent type IV collagen. There was no evidence that type I or type IV collagen regulated apoptosis in MIA PaCa-2 or Panc-1 cells.

It is not the intention of this discussion to overstate the significance of these results. The findings are interpreted in context of the following practical considerations.

1. It is acknowledged that inducing apoptosis in pancreatic cancer cell lines is difficult and this is reflected by a paucity of studies of apoptosis in pancreatic cancer. Apoptosis is a stochastic process and in these experiments, high doses of 5-FU were required to cause widespread apoptosis over short periods, which risked overwhelming even quite powerful anti-apoptotic signals. The process of apoptosis is a relatively rapid event and measuring synchronous apoptosis rather than a continuous low rate of apoptosis considerably increases the 'signal to noise' ratio and therefore practical feasibility of studying apoptosis. Moreover, in the face of a strong apoptotic signal, the effects of physiological and saturable signaling pathways might be expected to be modest. A compromise accounting for these practical problems took time to refine and thus allow confident identification or exclusion of apoptosis being regulated by extracellular matrix.
2. Pancreatic cancer cell lines were originally clonally selected by surviving isolation and early culture on TCP, meaning their phenotype was particularly suited to growth on TCP. The aim of these experiments was to identify changes in apoptosis between cells cultured on collagen and TCP, which despite being on different matrices, were anchored. The importance of anchorage to pancreatic cancer cells has recently been emphasized in a study that showed fibronectin and laminin reduced apoptosis/necrosis of MIA PaCa-2 and Panc-1 cells compared to cells cultured on polyHEMA. PolyHEMA prevents attachment of cells to TCP and thus allowed comparison of adherent cells with non-adherent cells. Extracellular matrix was accordingly concluded to protect pancreatic cancer cells from apoptosis, when perhaps it was really anchorage. Interestingly type I collagen did not confer the same protective effect as fibronectin and laminin, which is presumably related to the relatively inefficient adhesion of the MIA PaCa-2 cells to type I collagen (the effect of type I collagen on Panc-1 cells was not investigated) (Vaquero et al., 2003).

3. The use of primary cultures of pancreatic cancer cells was considered to try and overcome some of the problems engendered by studying cells with a robust phenotype. However, it is notoriously difficult to isolate cells from pancreatic cancer because of the desmoplastic reaction and it was therefore deemed an impractical solution.

Positive findings in this area of research should therefore be valued and considered in the context of a tumour *in vivo* where multiple cellular phenotypes are present. There has been little investigation of the functional consequence of the desmoplastic reaction and these experiments indicate the potential for pancreatic cancer cells to derive chemoresistance from the predominant constituent of the enveloping desmoplasia.

Treatment of the pancreatic cancer cells with 5-FU led to the membrane blebbing, nuclear condensation and fragmentation, increased caspase-3 activity and PARP cleavage, all signs of apoptosis. Pancreatic cancer cells undergo anoikis but in these experiments, it was clear in the cells treated with 5-FU that the apoptotic process had been initiated prior to detachment (e.g. membrane blebbing, see Fig 6.1) (Vaquero et al., 2003).

Evidence that type I collagen regulated apoptosis of AsPC-1 cells came from the study of PARP cleavage in cultures of AsPC-1 cells that had been treated with 5-FU. Studying PARP overcame the problem of studying a transient event, because cleaved PARP accumulated with time, allowing the cumulative effect of 5-FU to be measured. The study of PARP expression demonstrated that type I collagen and to a lesser extent type IV collagen, conferred protection to AsPC-1 cells treated with 5-FU. This appeared to be the case when studying the cell cultures with light microscopy prior to preparing protein lysates. The observations were clarified by demonstrating that AsPC-1 cells, which had become detached from the culture surface were apoptotic, with nuclear condensation and fragmentation. The apoptotic nature of the cells was confirmed when PARP expression was studied in the adherent and detached fractions of cells, which clearly demonstrated the detached cells expressed cleaved PARP. This provided a simple model in which counting the adherent and detached cell fractions allowed quantification of rates of apoptosis (Diaz et al., 2000). In the absence of phagocytic cells within the cultures, the apoptotic bodies accumulated, meaning that cumulative data could be obtained with lower doses of 5-FU. In keeping with the studies of PARP cleavage, this assay

demonstrated the protective effect of type I collagen was overcome by higher does of 5-FU. The doses of 5-FU used in these experiments were relevant. Serum concentrations of up to 130μg/ml of 5-FU are achievable in patients undergoing treatment (Dollery, 1999). The concentration of the drug achieved in the center of a solid tumour is likely to be less due to relative hypovascularity and whether it efficiently penetrates the abundant collagen remains unknown (Rang et al., 2001). Hence although the dose of 5-FU was less than 130μg/ml, it is probably analogous to what tumour cells are exposed to *in vivo*.

It is not clear why the relative changes in apoptosis were not detected by FACS analysis. FACS analysis of PI stained cells relies on the conversion of high molecular weight DNA fragments into low molecular weight fragments (oligonucleosomal laddering) by specific enzymes (Lecoeur, 2002). AsPC-1 cells, in common with other adenocarcinoma cell lines (e.g. MCF-7 breast cancer cells), may not possess these enzymes to mediate this process and as such would not reliably detect cells containing <2n DNA (explaining why the absolute rate of apoptosis detected in this assay was less than half that by counting) (Lecoeur, 2002). Furthermore, it studies the relative proportion of cells rather than the absolute numbers and therefore does not account for proliferation. These two factors may have made the reduction in apoptosis too small to measure by this technique. The caspase-3 assay was not applicable to studying matrix regulation of apoptosis because high doses of 5-FU were required to induce measurable caspase activity.

The bcl family of proteins control the release of cytochrome C from mitochondria, which is a key event in the apoptotic cascade that commits cells to death. It was clear from studying expression of the Bcl-2 homologues that they were subject to regulation in AsPC-1 cells treated with 5-FU. The principle changes that promoted apoptosis appeared to be an increase in Bak expression coupled to a reduction in Bcl_{XL} expression. The only consistent evidence of matrix regulation of these homologues was in mcl-1. Expression of mcl-1 was maintained relative to TCP and type IV collagen and this was likely to be an important factor in the relative resistance to apoptosis of AsPC-1 cells cultured on type I collagen. Bcl-2, Bcl_{XL}, mcl-1, bax and bak have all been identified *in vivo* by immunohistochemistry in pancreatic cancer (Friess et al., 1998; Virkajarvi et al., 1998; Evans et al., 2001). Strong Bax expression has been demonstrated to correlate with increased patient

survival time, whilst strong Bcl_{XL} expression with a reduced survival time (Friess et al., 1998; Evans et al., 2001). They are important in regulating chemosensitivity *in vitro.* The ratio of bax:bcl_2 predicts sensitivity to 5-FU and gemcitabine (Shi et al., 2002). Repeated exposure to these agents leads to induction of bcl_{XL} and mcl-1 expression and reduced chemosensitivity (Shi et al., 2002). Furthermore, bcl_{XL} anti-sense therapy has been shown to increase the sensitivity of pancreatic cancer cells to gemcitabine, whilst over expression of Bcl_2 leads to chemoresistance (Xu et al., 2001; Bold et al., 1999). Signalling via MAP/ERK pathway has been identified as important in regulating the expression of Bcl_2 homologues in pancreatic cancer cells (Boucher et al., 2000; Boucher et al., 2001). Constitutive activation of the PI3K/AKT signalling pathway has also been shown to inhibit apoptosis of pancreatic cancer cells (Bondar et al., 2002). Although the role of integrins has not been conclusively demonstrated in mediating the growth effects of collagen in this thesis, it has been shown that phosphorylation of protein tyrosine kinases (following ligation of integrins) is an important mechanism underlying chemoresistance derived from extracellular matrix (Sethi et al., 1999). Fas/TRAIL mediated apoptosis has been shown to be ineffective in pancreatic cancer cells through mechanisms such as failure of intracellular signalling pathways and strong expression of decoy receptors for Fas ligand (Ozawa et al., 2001; Kornmann et al., 2000; Ibrahim et al., 2001; Elnemr et al., 2001).

Very little is known about heat shock proteins in pancreatic cancer. HSP 70 is expressed at higher levels in pancreatic cancer, at mRNA level at least (Gress et al., 1994). It was expressed by AsPC-1 cells but was not induced by treatment with 5-FU or regulated by collagen in AsPC-1 cells. HSPs appear to play a more important role in acute pancreatitis (Rakonczay, Jr. et al., 2003).

Collagen type I and type IV did not consistently regulate changes in proliferation or apoptosis with respect to TCP. However, whether type I collagen supported growth or reduced apoptosis of the cancer cells, the effect on the cancer cell phenotype was the same, it manifested itself as a long-term survival advantage in the clonogenic assays. These assays are essentially an *in vitro* chemosensitivity assay and are analogous to a cycle of chemotherapy *in vivo* and as such provide powerful evidence of a biological effect (Steel, 2001). The resistant cancer clone is selected for by the chemotherapy and may repopulate the tumour, causing recurrent disease. Proliferation and apoptosis are two processes that are intimately linked and it is a combination of both that is measured by clonogenic assay (Evan and

Vousden, 2001). The growth and survival signals that a cell is receiving at the time of an injurious stimulus, dictates its fate. These pancreatic cancer cells are naturally equipped to resist cellular injury leading to apoptosis (e.g. p53 mutations, activating k-ras mutations). Type I collagen promotes this phenotype. Mechanisms underlying this include evidence of regulation of the bcl-2 homologues, particularly mcl-1, that would confer resistance to apoptosis. Amongst the cell cycle regulators that were studied, there was no evidence that cyclin D_1, p21 or p27 were subject to regulation in these tissue culture models. There are, however, numerous other cyclins and cyclin dependent kinases that promote cell growth and others that abrogate proliferation. They are subject to subject to very complex interactions and temporal control. This has contributed to the inability to identify the molecular control of growth stimulation/inhibition derived from collagen in the course of these experiments. Despite not demonstrating specific regulation of specific proteins, there will inevitably be changes in expression and regulation of molecules regulating the cell cycle, which in combination with regulation of bcl-2 homologues would augment the innate chemoresistance of pancreatic cancer cells.

Conclusions

Type I collagen that is abundant in the desmoplastic reaction, has the potential to inhibit apoptosis in pancreatic cancer cells. The combination of growth and survival promoting qualities of type I collagen lead to a long-term survival advantage in 3 pancreatic cancer cell lines.

Chapter 7

Expression of Extracellular Matrix Metallaloproteinase Inducer in Pancreatic Cancer

Introduction

Extracellular Matrix MetallaloPRoteinase INducer (EMMPRIN (also known as CD147 and basigin) is a novel cell surface molecule that has been postulated to play an important role in the interactions of malignant and stromal cells in tumours. It is a member of the immunoglobulin superfamily and is expressed by most epithelial cells. Expression of EMMPRIN by malignant epithelium is reportedly increased. Functionally it has been shown to stimulate secretion of MMPs by stromal cells, which may inappropriately facilitate invasion of cancer cells (Zucker et al., 2000). Nothing is known about its expression in pancreatic cancer. Because the interaction between PSC and pancreatic cancer cells contributes to the desmoplastic reaction a number of experiments were performed to determine whether EMMPRIN is expressed in pancreatic cancer.

Hypothesis

EMMPRIN is expressed in pancreatic cancer

Aims

1. Determine if EMMPRIN is present in the normal pancreas and ductal adenocarcinoma of the pancreas and characterize its expression.
2. Determine if pancreatic cancer cell lines express EMMPRIN.

Methods

Western analysis of EMMPRIN expression was conducted exactly as described in Chapter 2, with the following exceptions. The pancreatic cancer cells were lysed in Dignan A buffer (10mM HEPES, 10mM KCl, 0.2% NP40, 1.5mM $MgCl_2$, 0.5mM DTT) and not clarified by centrifugation prior to electrophoresis. Secondly the nitrocellulose membranes were blocked with 10% low fat milk w/v TST and 1% low fat milk w/v TST was used as the antibody diluent.

The immunohistochemisty for EMMPRIN relied on the protocols described in Chapter 3. A series of pilot experiments determined that the best results were obtained without using antigen retrieval techniques. The blocking stages needed to be extended from 10 to 30 minutes to inhibit non-specific binding. Specificity of the

primary antibody and secondary antibodies was ensured in 3 ways. The EMMPRIN antibody (clone N-19, Santa Cruz) was pre-incubated with its specific blocking peptide. The protocol was also repeated with isotype matched non-immune IgG (Sigma) and plain tris saline (TS), in place of the primary antibody. The final protocol employed the primary antibody diluted 1:400 v/vTS and the secondary antibody (Dako) diluted 1:200 v/vTS. A total of 18 sections were stained from patients who had undergone pancreaticoduodenectomy.

Results

1. Expression of EMMPRIN by pancreatic cancer cell lines

EMMPRIN expression amongst the pancreatic cancer cell lines was studied by Western analysis (method and antibodies listed in Chapter 2). It is a glycoprotein, which occurs in a non-glycosylated form (30kDa) and when fully glycosylated has a molecular weight of 57kDa. It expression was compared to that of a bladder cancer cell line, known to express EMMPRIN (Fig. 7.1). By this method, all 3 pancreatic cancer cell lines have been shown to express EMMPRIN.

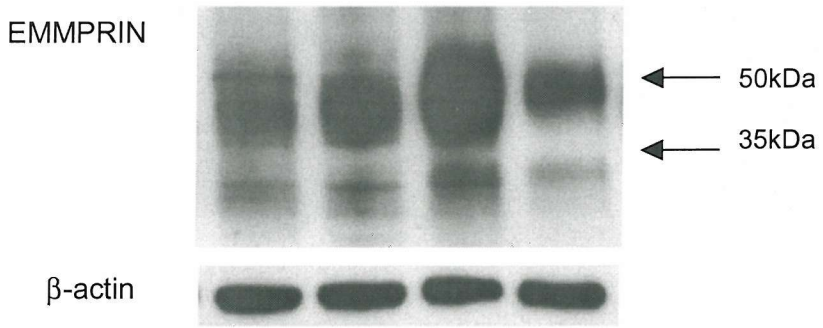


Figure 7.1
Western analysis of EMMPRIN expression in pancreatic cancer cell lines
Protein lysates were prepared from the pancreatic cancer cells under normal conditions and 25μg protein was subjected to SDS-PAGE. The bands represent the non-glycosylated form at 30kDa and the glycosylated form around 50kDa, as identified by a polyclonal antibody. From left to right: Panc-1, AsPC-1, MIA PaCa-2 and T24 bladder cancer cell line (positive control). β-actin was re-probed from the same membrane as a second loading control. There was no non-specific binding if the antibody was pre-incubated with its specific blocking peptide. The image (obtained using autoradiography) was representative of 3 independent experiments.

2. Expression of EMMPRIN in PDAC

Histological sections from 18 patients who had recently undergone pancreaticoduodenectomy in Southampton General Hospital were selected at random. Eleven patients had undergone surgery for PDAC and 7 further patients for

other peri-ampullary neoplasias: common bile duct tumours (2), duodenal cancer (3), duodenal adenoma (1), ampullary tumour (1). The normal pancreas, removed as part of these *en-bloc* resections, were used for control purposes. The sections were analysed with Dr. Adrian Bateman (Consultant Histopathologist) and the results are summarized in the table 7.1 and 7.2 below. It became clear from studying the controls that normal ductal epithelium demonstrated constant strong staining in all specimens, whereas stromal elements did not stain at all (Fig 7.2A). A simple scoring system was adopted based on this feature, whereby the staining intensity in normal ducts was ascribed ++ and stromal staining -. This allowed a simple, systematic description of EMMPRIN staining in the controls and pancreatic cancer specimens.

Case	Diagnosis	Normal Pancreas			
		Acinar Cells	Centroacinar Cells	Duct Cells	Connective Tissue
1	CBD	-	+	++	-
2	Normal	-	++	++	-
3	DC	-	++	++	-
4	FAP	-	+	++	-
5	DC	-	++	++	-
6	DC	-	++	++	-
7	CBD	-	+	++	-

Table 7.1
Immunohistochemical expression of EMMPRIN in the normal pancreas
Normal pancreas was removed during pancreaticoduodenectomy for peri-ampullary neoplasia (CBD=adenocarcinoma of the common bile duct, DC=duodenal carcinoma, FAP=duodenal adenomas associated with familial adenomatous polyposis, Normal=no pathological lesion demonstrated). The sections were stained for EMMPRIN and the intensity scored in comparison to normal ductal epithelium and stroma as described above.

The pattern of staining was consistent. The 'outer' acinar epithelium did not stain for EMMPRIN. The junction between acinar cells and ductal epithelium is occupied by centroacinar cells, which mediate the transition between the two. These begin to stain positively but less so than the larger ducts in 3 of 7 cases. Hence there would appear to be a gradual transition between acinar cell and duct cell that was clear in approximately half the cases. An example of this is shown in Fig. 7.2B. The pattern of staining was predominantly cytoplasmic in all cell types. This consistent pattern

of staining was also seen in the normal pancreas away from the malignant areas in the pancreatic cancer slides and a similar approach was therefore adopted in grading the staining of the malignant epithelial cells. In each case the intensity of the staining was compared to that of normal ductal epithelium within each sample (Table 7.2).

Case	Benign Pancreas				Malignant Areas	
	Acinar Cells	Centroacinar Cells	Duct Cells	Connective Tissue	Malignant Epithelial Cells	Desmoplastic Tissue
1	-	+	++	-	++	-
2	-	++	++	-	+	-
3	-	++	++	-	-	-
4	-	++	++	-	-	-
5	-	+	++	-	++	-
6	-	++	++	-	++	+
7	-	++	++	-	++	-
8	-	++	++	-	+	-
9	-	+	++	-	-	-
10	-	++	++	-	++	-
11	-	++	++	-	++	-

Table 7.2
Immunohistochemical expression of EMMPRIN in ductal adenocarcinoma of the pancreas
Eleven sections were stained for EMMPRIN and the intensity was scored in comparison to normal ductal epithelium and stroma as described above.

In 5 cases EMMPRIN staining in the malignant epithelium was either absent or weak relative to the normal ductal epithelium. This is clearly demonstrated in Fig. 7.2C, which has benign and malignant ducts in the same field. In the remaining 6 cases the intensity of staining was equivalent to that seen in the normal ducts (Fig. 7.2D). It was not possible to reliably correlate the degree of staining in the malignant cells with the degree of desmoplasia. The main reason for this was the peri-tumoural pancreatitis, which itself was characterised by fibrosis, thus making the edge of the tumour ill-defined. The staining was predominantly cytoplasmic but

in general it was more intense at the periphery of the cell. There was also intense staining of the duodenal epithelium in all cases (Fig. 7.3).

Discussion

The role of EMMPRIN in stimulating MMP expression by stromal cells is now well established. It is reported that interactions in *in vitro* co-culture models of malignant and stromal cells in malignant melanoma, breast cancer and glioma led to up-regulated expression of a variety of MMPs and this was mediated by EMMPRIN (Sameshima et al., 2000; Kanekura et al., 2002; Taylor et al., 2002). This phenomenon did not occur in co-culture models of PSC and pancreatic cancer cells, which left no substantial rationale for hypothesising and therefore testing the biological role of EMMPRIN in the tissue culture models described in Chapter 4. There is no experimental data regarding EMMPRIN expression in PDAC and as a mediator with potential impact in the desmoplastic reaction, its expression was studied.

In common with studies of malignancies in the lung, breast, larynx, skin, bone and ovary, EMMPRIN was expressed in pancreatic cancer (Caudroy et al., 1999; Rosenthal et al., 2003; Kanekura et al., 2002; Si et al., 2003; Davidson et al., 2003). In contrast to the malignant cells in lung, breast, melanoma and larynx, which all express EMMPRIN more strongly than their benign epithelial precursors, pancreatic cancer cells express it at a similar level or more weakly than normal ductal epithelium (Caudroy et al., 1999; Kanekura et al., 2002; Rosenthal et al., 2003). In context of the function of EMMPRIN this finding is consistent with the florid desmoplastic reaction in pancreatic cancer, where the balance of matrix turnover favours matrix accumulation. With the exception of breast cancer these other tumours are not characterized by a marked stromal reaction, particularly the tumours derived from squamous epithelia which appear to express EMMPRIN most strongly (Caudroy et al., 1999). Unfortunately, it was not possible to satisfactorily correlate EMMPRIN expression with the degree of desmoplasia, which would have given support to this observation.

EMMPRIN (CD 147, basigin) is proving to be a rather complex and multifunctional protein. It is a member of the immunoglobulin superfamily, encoded on chromosome 19, which when knocked out leads to a phenotype characterized by neurological abnormalities and sterility (Muramatsu and Miyauchi, 2003). It is predominantly expressed in the cell membrane but has also been identified in

intracellular vesicles (and cytoplasm in malignancy and inflammatory conditions) (Taylor et al., 2002; Caudroy et al., 1999; Konttinen et al., 2000). It is subject to homophillic interaction, in the context of both homo and heterotypic interactions and approximately 2-3% occurs in a secreted form (Sun and Hemler, 2001; Taylor et al., 2002). It activates the phospholipase A_2/5-lipoxygenase pathway, which leads to release of MMPs (Taylor et al., 2002). As well as playing an important role in malignancy, it is also expressed in high levels in rheumatoid arthritis and inflammatory tissue associated with venous leg ulcers (Konttinen et al., 2000; Tomita et al., 2002; Norgauer et al., 2002). Furthermore, it is reported to participate in HIV infection and act as a co-factor of sorts to the monocarboxylate transporter (and as such is implicated in non-insulin dependent diabetes) (Muramatsu and Miyauchi, 2003; Zhao et al., 2001).

Investigation of the function of EMMPRIN has been conducted in a variety of culture models. Studies of its role in regulating MMP expression have shown that its involved in up-regulation MMP-2 (in every model), MMPs 1,3 and 9 and MT-1/2 MMPs (Sameshima et al., 2000; Kanekura et al., 2002). The effects of EMMPRIN seem to be most pronounced when cells are co-cultured (as opposed to supernatant transfer models), presumably because the majority is membrane bound (Kanekura et al., 2002; Bordador et al., 2000). The use of anti-EMMPRIN antibodies (and anti-sense therapy) has been shown to reduce invasion in a diversity malignant cells (by virtue of reducing MMP expression), reflecting the homotypic stimulation (Bordador et al., 2000; Yang et al., 2003; Li et al., 2003). Heterotypic stimulation between malignant and stromal cells has been reproduced using recombinant EMMPRIN and also abrogated with blocking antibodies and immunodepletion techniques (Sun and Hemler, 2001; Bordador et al., 2000; Yang et al., 2003; Taylor et al., 2002). As well as stimulating the release of MMPs, EMMPRIN has also been demonstrated to bind MMP-1 to the cell surface (Guo et al., 2000). This may be significant as tumour spread may be facilitated with low levels of EMMPRIN expression in the context of the desmoplastic reaction. When studied *in vivo*, EMMPRIN transfected breast cancer cells a phenotype characterized by greater tumour burden and invasion, which was attributed to greater gelatinase expression (Zucker et al., 2001).

Conclusion

EMMPRIN is expressed in the pancreas but at equivalent or reduced levels PDAC. In context of its known functions this is consistent with the marked desmoplastic reaction. There was no evidence that pancreatic cancer cells stimulated MMP production by PSC *in vitro* and so the significance of this remains to be determined.

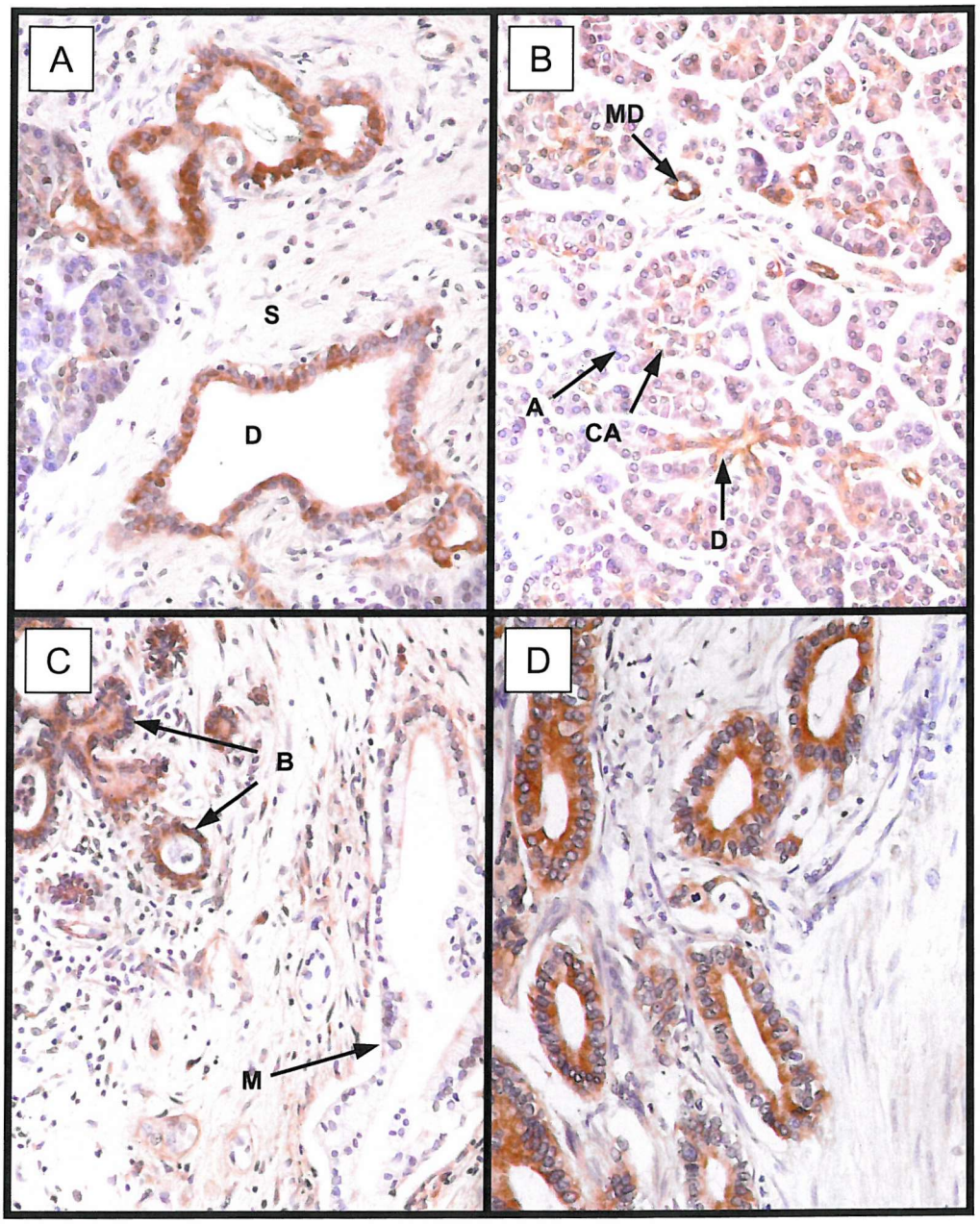


Figure 7.2
Normal Pancreas and PDAC stained for EMMPRIN

A. Normal pancreas (S: stroma, D: duct). **B.** Normal pancreas (A: acinar cell, CA: centroacinar cell, D: duct system, MD: major duct). **C.** PDAC (B: benign ducts, M: malignant epithelium). **D.** PDAC

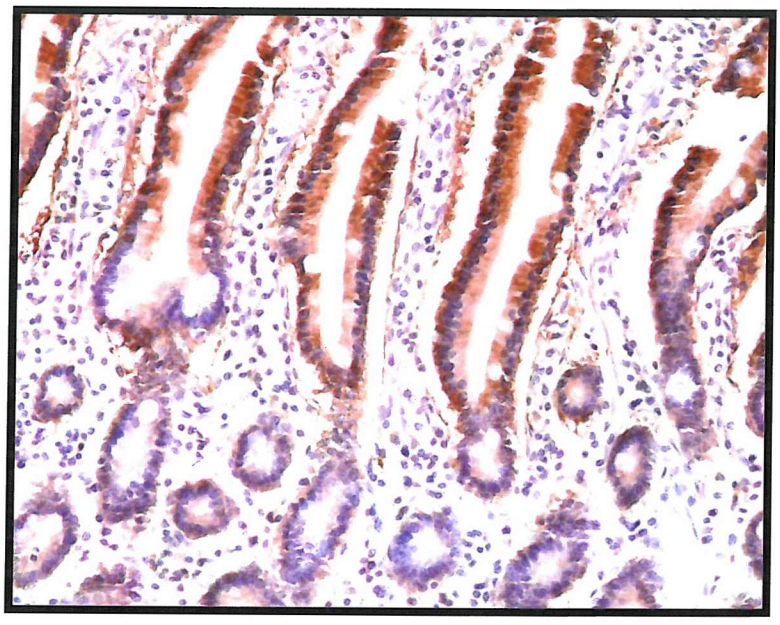


Figure 7.3
Duodenum stained for EMMPRIN

Chapter 8
Discussion

1. A hypothetical model of malignant-stromal interactions in pancreatic cancer

In the course of investigating the origin and role of the desmoplastic reaction in pancreatic cancer, a hypothetical model of malignant-stromal interactions in pancreatic cancer has emerged and the potential consequences of this are illustrated in figure 8.1. The numbers in the following discussion refer to fig 8.1, which draws together the experimental findings.

1. Although the cellular origin and the roles of metaplasia, genetic and environmental factors remain undetermined, ductal adenocarcinoma of the pancreas is characterised by cells with a ductal phenotype that form aggressive tumours with a marked stromal component.
2. Three pancreatic cancer cell lines consistently stimulated proliferation of primary cultures of PSC in supernatant transfer experiments. This finding provides potential mechanism to explain the large numbers of PSC identified in the stromal reaction *in vivo*.
3. PSC were found to secrete collagen in excess of the malignant cells *in vitro* and the increased numbers of PSC found in pancreatic cancer are likely to be responsible for the excessive deposition of fibrillar collagen. One of the three cell lines (AsPC-1) also stimulated transcription of procollagen I and increased total collagen synthesis by PSC. This is likely to have been related to the high level of $TGF\beta_1$ secretion by the cell lines.
4. The cultured PSC were found to secrete TIMP-1 in excess of MMP-2 and this balance would potentiate matrix accumulation within the desmoplastic reaction *in vivo*. The expression of MMPs and TIMPs by cancer cell lines was generally weak and inconsistent and as does not provide strong evidence to speculate about their role in matrix regulation *in vivo*.
5. Ducts and acini are normally surrounded by type IV collagen in the normal pancreas. This defined pattern is lost in pancreatic cancer and type I (and III) collagen dominates the tumour stroma. This process may be contributed to in part by MMP-2 (secreted by PSC and to a lesser extent cancer cells) mediated basement membrane degradation. (Moreover it may be potentiated by membrane bound MMPs (e.g. MT-1 MMP), also made by PSC but not effectively inhibited by TIMP-1).

6. Perhaps a more important factor in the marked accumulation of fibrillar collagen relative to type IV collagen in the desmoplastic reaction is the propensity for PSC to secrete fibrillar collagen.
7. Type I and III collagen stimulated proliferation of all 3 cell cancer cell lines relative to type IV collagen *in vitro* and the dominance of these collagens in the desmoplastic reaction would therefore be expected to promote growth of the cancer cells *in vivo*. The metastatic AsPC-1 cells were the only line not subject to inhibition by type IV collagen, and this phenotype may contribute to metastasis.
8. Culture of AsPC-1 cells on type I collagen also increased their resistance to 5-FU induced apoptosis, relative to TCP. There was no evidence that matrix regulated apoptosis in Panc-1 and MIA PaCa-2 cells. Type I collagen did however, consistently preserve clonogenic potential of all 3 cell lines (relative to TCP) after treatment with 5-FU, which is probably more important in the context of chemo and radiotherapy in patients. This finding is likely due to regulation of proliferation and apoptosis by type I collagen. Although the exact molecular mechanisms underlying this were not clearly demonstrated during the course of these experiments, it is probable that collagen regulates cell cycle and Bcl-2 proteins. Furthermore, by analogy with hepatic stellate cells, type I collagen is also likely to promote PSC survival and activation (Issa et al., 2003).
9. These interactions, once initiated are presumably subject to an amplifying feed back loop, which contributes to the histological appearance and perpetuates the inexorable growth of pancreatic cancer *in vivo*.

It is acknowledged that immune cells (e.g. macrophages and lymphocytes) and matrix other that collagen (e.g. laminin, fibronectin) may also play a role in the phenotype of pancreatic cancer. However, the interaction between malignant cells, PSC and collagen (which would appear to be the main cellular and stromal commitments in pancreatic cancer) are deleterious.

2. The wound healing response and cancer

The wound healing response has evolved over millions of years and provides good defence against trauma but is ill equipped to eradicate cells that have undergone malignant transformation. Indeed there is increasing evidence that the inflammatory and wound healing stroma associated with cancer may promote tumourigenesis by secreting cytokines, promototing aniogenesis/lymphangiogenesis and secreting collagen (Coussens and Werb, 2002). It has long been supposed that the desmoplastic reaction is 'host' driven (which implies immune mediated) and is some form of defence (Seemayer et al., 1982). I have come to the conclusion that this is not correct, assuming the initial oncogenic process is discrete from subsequent tumourigenesis. Potential myofibroblasts (e.g fibroblasts/PSC) occupy a position beneath the basement membrane in the supporting interstitial tissue.
Myofibroblastic activity is increased at an early stage of tumourigenesis, even being associated with dysplastic epithelium (Schurch, 1999; Liotta and Kohn, 2001). Once the myofibroblastic phenotype is acquired, so the wound 'healing' begins, with matrix deposition and turnover: these are conducive conditions for growth of malignant cells. It is therefore possible that 'successful' neoplastic cells self-select by stimulating a stromal reaction that will subsequently support tumourigenesis or are selected for because they derive and advantage from the stromal reaction. Chronic inflammation is often complicated by malignancy, including in the pancreas (Farrow and Evers, 2002; Stevens and Lowe, 1995a). Perhaps this is because the wound healing has already begun, thereby providing a nurturing environment for oncogenesis.

3. Potential for 'Stromal Therapy'

The importance of studying the desmoplastic reaction in pancreatic cancer is well recognized yet it remains in its infancy (Kern et al., 2001). The report of a recent American Association of Cancer Research meeting devoted to 'Proteases, Extracellular Matrix and Cancer' highlights this anomaly (Wall et al., 2003). Many advances in understanding the field of cell-matrix cancer biology have been made. Furthermore, new techniques and models to investigate such interactions are being developed. However, the widespread development of therapeutics as a result of this type of research appears to be some way off.

The great hope in this field was MMP inhibitors, which demonstrated encouraging anti-tumour properties *in vitro* and in animal models (Chau et al., 2003). For a variety of reasons they have been disappointing in the treatment of patients. This

may have been related to study design, often recruiting patients with advanced tumours and using survival as end points (Chau et al., 2003). They have failed to be beneficial in the treatment of pancreatic cancer (Bloomston et al., 2002a). However, there is continued optimism that blockade of single MMPs or TIMP-1 over-expression (using gene transfer technology) may yet prove encouraging (Baker et al., 2002). It is becoming increasingly clear that MMPs and their inhibitors play extremely complex roles in tumour biology (as illustrated by this study) and the potential growth promoting properties of TIMPs in particular should be recognised when designing therapeutics (Baker et al., 2002). It is clear from the experimental data presented in this thesis that the interactions between stroma and cancer occurs at many levels. Inhibiting one process that promotes the malignant phenotype (e.g. MMP inhibition) may enhance another (e.g. accumulation of growth promoting extracellular matrix).

MMPIs may have improved efficacy if used in combination with therapies that aim to inhibit type I collagen synthesis and the development of fibrosis. Pirfenidone [5-methyl-1-phenyl-2-(1H)-pyridone] inhibits the development of fibrosis, although its exact mechanism of action remains undetermined (Lindor et al., 2003). It reduces fibrosis of bleomycin induced lung injury (Iyer et al., 1999a). Furthermore it abrogates liver fibrosis in animal models by inhibiting proliferation and collagen synthesis amongst HSC (Tada et al., 2001; Di Sario et al., 2002). It reduces expression of TGFβ, procollagen gene expression and reduces inflammatory cell infiltrate (Iyer et al., 1999b; Iyer et al., 1999a). Another approach would be to block the effects of TGFβ. In animal models of hepatic cirrhosis, adenovirally delivered dominant negative truncated TGFβ II receptor transgenes were delivered by adenoviral vectors via the portal vein (Qi et al., 1999). This led to a dramatic reduction in liver fibrosis (Qi et al., 1999). Similar protection from liver injury was conferred by injection of soluble TGFβ II receptor (George et al., 1999). Transient treatment of this nature had no side effects in the short term but long-term effects are unknown (Boivin et al., 1995). It would be logical to test the therapeutic potential of this type of treatment in pancreatic cancer. Unfortunately pancreatic cancer research is constrained by the absence of a good animal model (see below).

Gene therapy, mentioned above in the context of TIMP-1 and TGFβ, is another area in which malignant:stromal interactions may be specifically targeted in the future. The two main approaches employ either the immune system or directly

targets the malignant cells, reviewed by Tseng and Mulligan (Tseng and Mulligan, 2002). Immune based therapy that may prove beneficial in pancreatic cancer relies on antigen presenting cells (e.g. dendritic cells) to stimulate cellular and humoural immunity against tumour specific antigens, thus amplifying the response. Mutated K-ras, p53 and MUC-1 are candidate antigens that have been used in nude mice models of pancreatic cancer with mixed success. The other approach requires widespread transduction of genes in tumour cells using viral transfection. The aim of this therapy includes restoration of wild type proto oncogenes or tumour suppressor genes (e.g. wild type p53) to tumour cells or to disable oncogenes (e.g. via transfection of K-ras antisense plasmids) both of which have shown promise in *in vitro* models of pancreatic cancer. The difficulty of translating this type of research into success *in vivo* is efficiently transfecting all tumour cells and relying on the restoration of a single gene to restore a non-malignant phenotype (Tseng and Mulligan, 2002). However, this type of therapy may well compliment conventional chemotherapy.

The use of gene arrays and proteomics to study gene expression in PDAC will help to identify novel targets for gene therapy. A recent example of this is in pancreatic cancer was the discovery of Claudin-4, an integral constituent of tight junctions (Michl et al., 2003). Over expression of this gene inhibited the malignant phenotype by reducing invasion and metastasis. There is increasing interest in gene expression in the malignant:stromal interface in pancreatic cancer and this type of research should identify other candidates (like TGFβ) which would potentially interfere with the formation of the desmoplastic reaction (Iacobuzio-Donahue et al., 2002b).

Integrins represent another potential therapeutic target. Blocking β_1 integrin inhibited proliferation of one pancreatic cancer cell line and β_1 integrins play important roles in other tumour phenotypes (Sethi et al., 1999; Bissell et al., 2002). The problem of β_1 integrins as a therapeutic target is that they are common to most cells of the body. $\alpha_V\beta_3$ integrin may provide a more specific target. It is not widely expressed in healthy tissue but has been demonstrated in a variety of malignancies. It is expressed by endothelial cells in developing blood vessels (e.g. in wounds) and has been implicated in tumour angiogenesis (as well as mediating cancer cell growth and invasion) (Tucker, 2002). $\alpha_V\beta_3$ has a wide variety of ligands, all of which represent potential therapeutics. Of particular interest in the context of

this work, are peptides derived from extracellular matrix. Endostatin is derived from the non-collagenous domain of type XV collagen and is anti-angiogenic in a number of animal tumour models but its biological effects are not fully understood (Wickstrom et al., 2003; Ortega and Werb, 2002). There is also interest in type IV collagen α_3(NC1) which has been shown to inhibit angiogenesis, cellular proliferation and MMP expression amongst others (Ortega and Werb, 2002). The RGD (Arg-Gly-Asp) peptide sequence is another $\alpha_V\beta_3$ ligand, a synthetic form of which reduces metastasis in an animal model of hepatocellular carcinoma by reducing adhesion, invasion and reducing MMP expression (Tsuchiya et al., 2002). Function blocking anti $\alpha_V\beta_3$ antibodies (Vitaxin^{TM}) have even reached clinical trials. They were well tolerated by patients and had a cytostatic effect in about half the patients with a variety of non-haematological malignancies (Gutheil et al., 2000). Although the role of integrins in pancreatic cancer cell growth were not clearly established in the course of my experiments, it is possible that they are critical in 3D culture (see below) and in time be a realistic therapeutic target in pancreatic cancer.

It is clear that extracellular matrix is as important to tumour phenotype as growth factors and cytokines and it is therefore vital to continue this type of research because without full understanding, the potential of promising therapeutic agents may not be recognised. Furthermore, low dose stromal therapy might potentially reverse subtle but critical imbalances in tumour-host signals that may enhance the effect of other more conventional agents (Liotta and Kohn, 2001).

4. Future Directions

The future of this type of research lies in studying the effect of extracellular matrix in 3-dimensions (3D) (Abbott, 2003). Indeed $40 million will be invested annually in studying the cellular microenvironment at the United States National Cancer. There is good evidence that 3-dimensional culture can profoundly affect cell growth and MMP expression the ability of malignant cells to move and grow in 3D may be central to their phenotype (Bissell et al., 2002). This is best illustrated by the behaviour of breast cancer cells.

The breast is subject to constant remodelling through puberty, pregnancy and menopause and as such is characterized by cells that are very responsive to their environment. Benign and malignant breast epithelial cells cultured in 2D have a very similar phenotype but with 3D culture in Matrigel, the benign cells polarize and

form regular glands whereas certain malignant cell lines form larger, irregular clusters (Bissell et al., 2002). Other studies have demonstrated it is possible to induce polarity in malignant breast epithelial cells with 3D culture. It was found that the development of polarity conferred resistance to a variety of apoptotic stimuli. This was subsequently abolished by blockade of β_1 integrins whereupon polarity was lost. Furthermore if benign cells were cultured in type I collagen gels, their polarity is reversed (i.e. the glands become inside out) (Bissell et al., 2002).

3D culture therefore profoundly effects gene expression in the breast and this is likely to be the same in the pancreas. Two important concepts that emerge from studies in breast and other tissues and should be considered when planning further investigation in this area.

1. Epithelial cells normally grow in 2D on basement membrane, although glands ultimately form 3D structures. Mesenchymal cells (or mesenchymally derived cells) occupy a position in the interstitium and normally exist in a genuinely 3D environment. Invading malignant cells may end up in this 3D environment but will often remain as irregular glandular structures.
2. Mesenchymal cells are in contact with interstitial matrix, whereas epithelial cells are adherent to basement membranes. It is likely interactions with each respective matrix regulate the normal behaviour of both cell types (Ingber, 2002). My results indicate that contact with the wrong matrix may promote a deregulated phenotype in the same way that type I collagen reversed polarity amongst breast cells (Bissell et al., 2002).

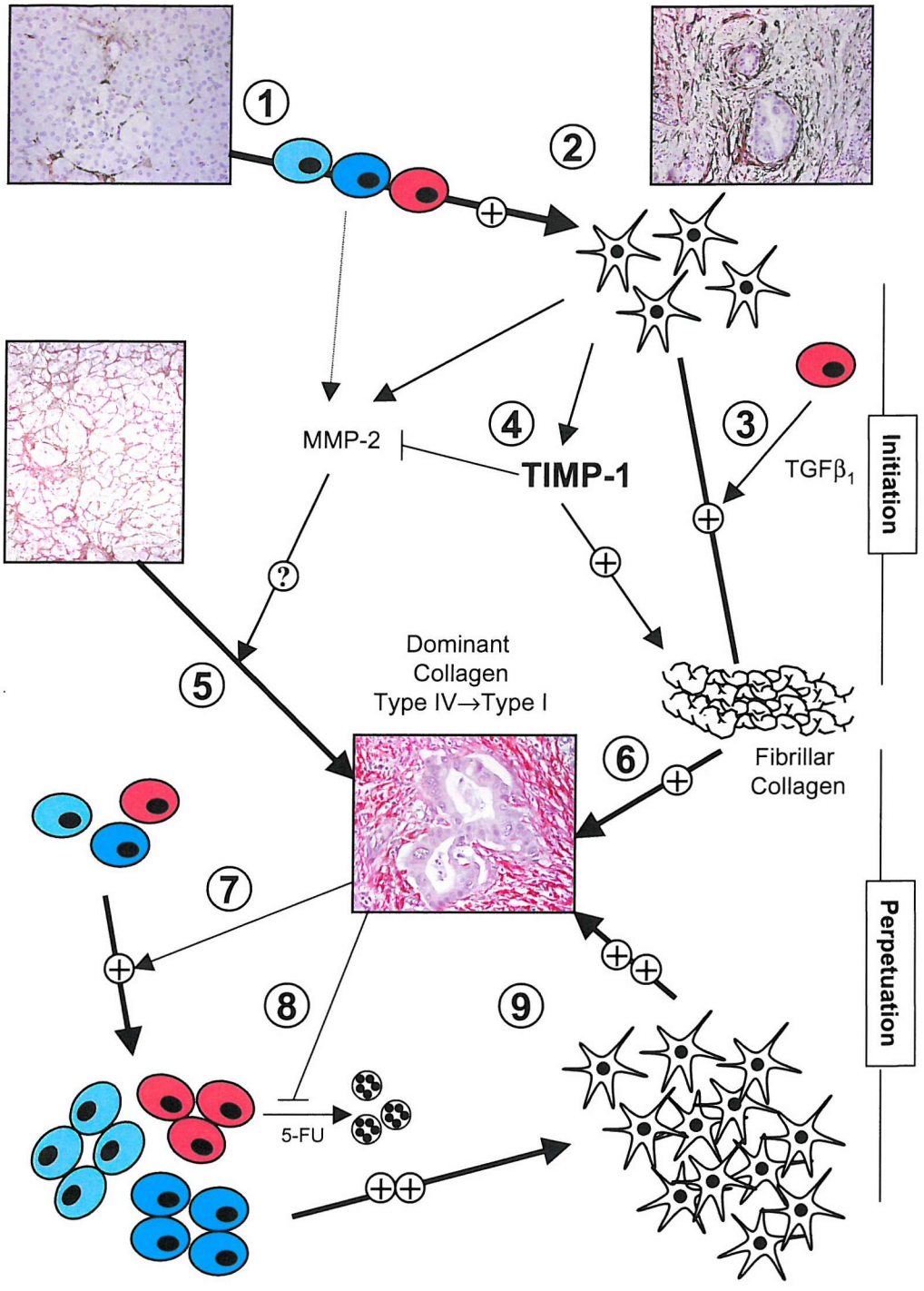
There are commercial 3D culture systems for collagen (e.g. Vitrogen) basement membrane (e.g. Matrigel) or synthetic neutral biological scaffolds (e.g. Puramatrix). Transferring techniques to these culture systems will be challenging.

Pancreatic cancer research is restricted by the lack of a good animal model (Lowy, 2003). The model that produces tumours most akin to human PDAC is the N-nitrosobis(2-oxopropyl)amine (BOP) induced tumours in the Syrian Golden hamster (Lowy, 2003). Research in this area is hampered by a paucity of knowledge of the hamster genome and the lack of availability of commercially available hamster reagents. There are no recognized transgenic animal models that develop PDAC. Progress in this field is further limited by a lack of knowledge of gene promoters in PDAC and the ongoing debate of the cellular origin of the disease (Pour et al., 2003). It would be possible to determine the effect of PSC in tumourigenesis by co-

injecting PSC and cancer cells into nude mice. This has provided interesting results in prostate and breast cancer research and would indicate how PSC may affect tumour forming efficiency and growth in pancreatic cancer (Olumi et al., 1999; Noel and Foidart, 1998). It would also permit the study of drugs like pirfenidone in an *in vivo* context.

5. Concluding Remarks

Ductal adenocarinoma of the pancreas has a greater stromal component than virtually any other tumour and has virtually the worst prognosis of any other tumour. This work contributes to the field of pancreatic cancer research by shedding new light on the contribution of the desmoplastic reaction to the malignant phenotype of pancreatic cancer. I hope this new understanding will stimulate further research in this field and ultimately lead to the development of new effective treatments for pancreatic cancer.



Legend

- MIA PaCa-2
- Panc-1
- AsPC-1
- PSC
- Apoptotic body

Figure 8.1
An Integrated Hypothetical Model
Diagram to illustrate how findings from *in vitro* models of pancreatic cancer may manifest themselves *in vivo* (the accompanying text provides an explanation via the numbers). Red indicates the metastatic cell line.

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Appendices

Appendix 1a

Integrin	Extracellular Matrix Ligand
$\alpha_1\beta_1$	collagen, laminin
$\alpha_2\beta_1$	collagen, laminin
$\alpha_3\beta_1$	laminin, thrombospondin-1, collagen, fibronectin
$\alpha_4\beta_1$	fibronectin
$\alpha_4\beta_7$	fibronectin
$\alpha_5\beta_1$	fibronectin
$\alpha_6\beta_1$	laminin, thrombospondin-1
$\alpha_6\beta_4$	laminin
$\alpha_7\beta_1$	laminin
$\alpha_8\beta_1$	fibronectin, tenascin
$\alpha_9\beta_1$	tenascin, collagen, laminin
$\alpha_{10}\beta_1$	collagen
$\alpha_{11}\beta_1$	collagen
$\alpha_v\beta_1$	fibronectin, vitronectin
$\alpha_v\beta_3$	vitronectin, fibronectin, von Willebrand Factor, tenascin, thrombospondin-1
$\alpha_v\beta_5$	vitronectin
$\alpha_v\beta_6$	fibronectin, tenascin
$\alpha_v\beta_8$	collagen, laminin, fibronectin, vitronectin, von Willebrand Factor

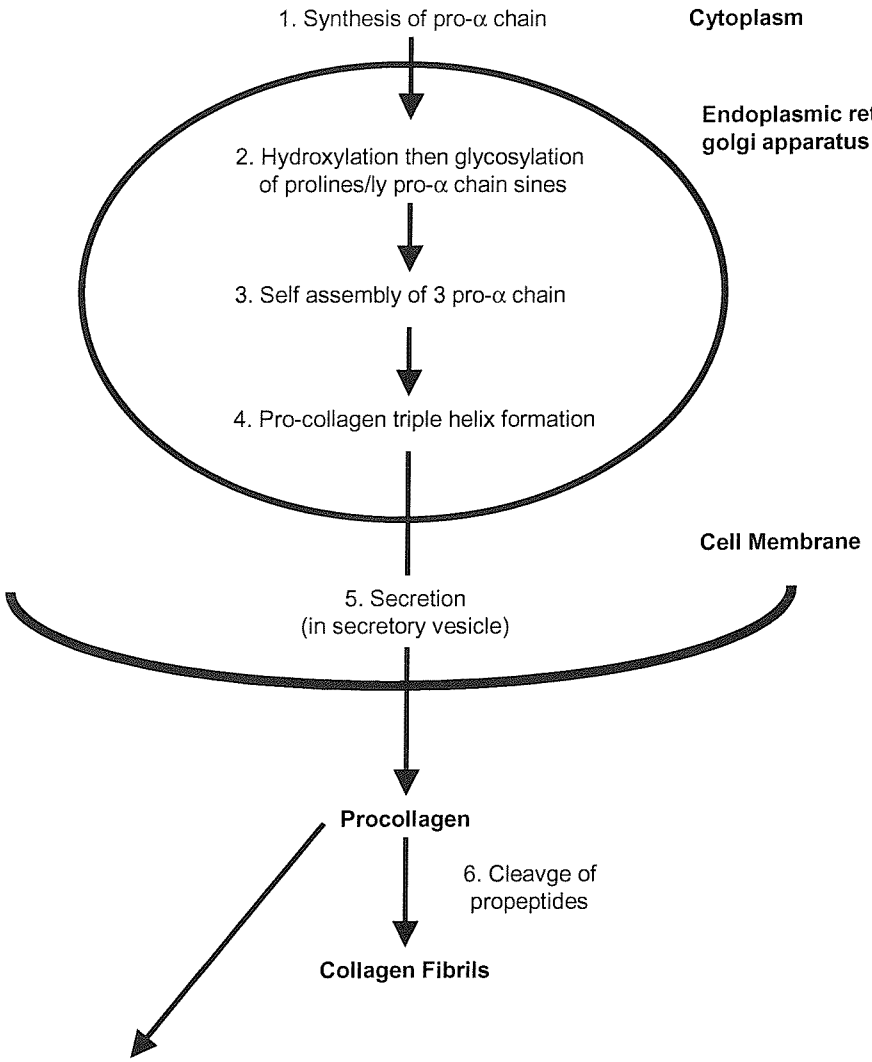
Table A1.1
Normal extracellular matrix ligands
(van der Flier and Sonnenberg, 2001)

Appendix 1b

	Type	Molecular Formula	Polymerised Form	Tissue Distribution
Fibrillar	I	$[\alpha1(I)]_2\alpha2(I)$	fibril	bone, tendon, ligaments, cornea, internal organs
	II	$[\alpha1(II)]_3$	fibril	cartilage, intervertebral discs, vitreous humour
	III	$[\alpha1(III)]_3$	fibril	skin, blood vessels, internal organs
	V	$[\alpha1(V)]_2\alpha2(V)$	fibril (with type I)	as for type I
	XI	$[\alpha1(IX)]\alpha2(IX)\alpha3(XI)$	fibril (with type II)	as for type I
Fibril-Associated	IX	$[\alpha1(IX)]\alpha2(IX)\alpha3(XI)$ with type II fibrils	lateral association	cartilage
	XII	$[\alpha1(II)]_3$ with some type I fibrils	lateral association	tendon, ligaments
Network-Forming	IV	$[\alpha1(IV)]_2\alpha2(IV)$	sheetlike network	basal laminae
	VII	$[\alpha1(IV)]_3$	anchoring fibrils	beneath stratified squamous epithelia

Table A1.2
Basic classification of common collagens
(Alberts et al., 1994)

Appendix 1c



Basement membrane collagens form by a similar process but unlike fibrillar collagens their terminal domains are not cleaved after secretion, which hinders side to side packing and fibril formation. Instead the terminal domains interact forming a sheet like network, that provides the structural basis of basement membranes (see figure 1.4). These terminal domains may have important biological functions.

Figure A1
The principles of collagen secretion
Adapted from (Alberts et al., 1994)

Appendix 2

Mycoplasma Testing

Cell lines were guaranteed mycoplasma free on purchase but this was rechecked after a stock had been generated and refrozen. This was done using a commercial Mycoplasma Testing Kit (ATCC) according to the manufacturers instructions. Sub-confluent cultures of the pancreatic cancer cell lines were lysed in the kit buffer and incubated at 95°C for 10 minutes. The lysate was then subjected to a 2 stage PCR process using the primers supplied (with 30 cycles of denaturing (94 °C/30sec), annealing (55 °C/30sec) and extension (72 °C/60sec) for each stage. The PCR products were separated on a 1% agarose gel and visualised using ultraviolet light (see chapter 3). The cells were confirmed to be mycoplasma free (Fig. A2).

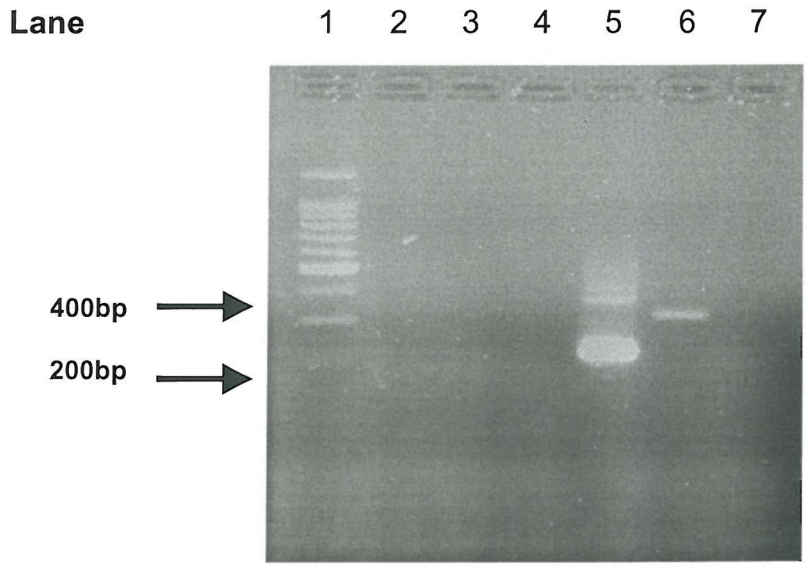


Figure A2
Demonstration that cell lines were mycoplasma free
The pancreatic cancer cell lines were confirmed mycoplasma free by excluding the presence of mycoplasma genomic DNA from cell cultures (Lane 1: 100 base pair ladder, 2: MIA PaCa-2, 3: Panc-1, 4: AsPC-1, 5: Acholeplasma laidlawii (supplied positive control), 6: Mycoplasma pirium (supplied positive control), 7: water (negative control). Image obtained using a Kodak digital camera.

In order to avoid mycoplasma contamination, cells were cultured in a designated mycoplasma free environment (where possible) and used for 20 passages only.

Appendix 3

Evidence supporting use of manganese ions in experiments with extracellular matrix

Manganese ions (Mn^{2+}) have been reported to enhance the constitutive activity of β_1 integrins and have been used to promote integrin function in this field of research previously (Sethi et al., 1999; Bazzoni et al., 1995). Pilot experiments were therefore undertaken to determine the effect of Mn^{2+} on the adhesion of pancreatic cancer cell lines (Fig. A3).

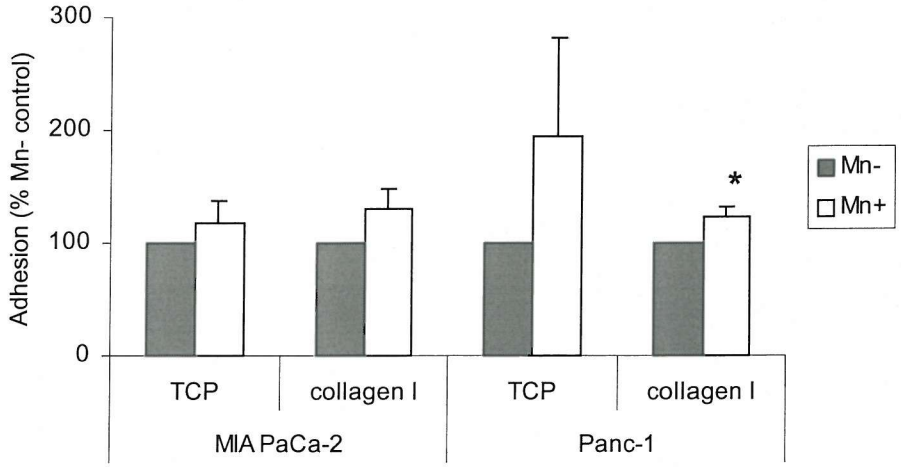


Figure A3
Effect of manganese ions on short-term adhesion of pancreatic cancer cell lines
$5x10^4$ MIA PaCa-2 or Panc-1 cells with (Mn+) or without (Mn-) 0.1mM Mn^{2+} added to the medium, were seeded into wells prepared as described in Chapter 2. After 45 minutes incubation at 37°C, the number of adherent cells was then quantified using methylene blue (see Chapter 5). The bars represent the mean of 3 independent experiments +/-SEM. Each is normalised to its own Mn- control that has been given an arbitrary value of 100% (*$p<0.05$ with respect to Mn- control).

This demonstrated that 0.1 mM Mn^{2+} appeared to consistently enhance the short-term adhesion of pancreatic cancer cells. Despite the increase being significant in only Panc-1 cells cultured on type I collagen, Mn^{2+} were routinely added to the media in all experiments using extracellular matrix, based on these findings. (These experiments were undertaken prior to the acquisition of AsPC-1 cells).

Appendix 4

The principles of TaqMan RT-PCR

PCR primers and probes are designed to detect a specific target region of a gene of interest. The probe is labeled with a fluorescent dye and a quencher preventing fluorescence. As polymerization occurs, the annealed probe is cleaved by Taq polymerase separating the fluorescent dye from the quencher. With each PCR cycle, more probe is cleaved until fluorescence reaches a detectable threshold.

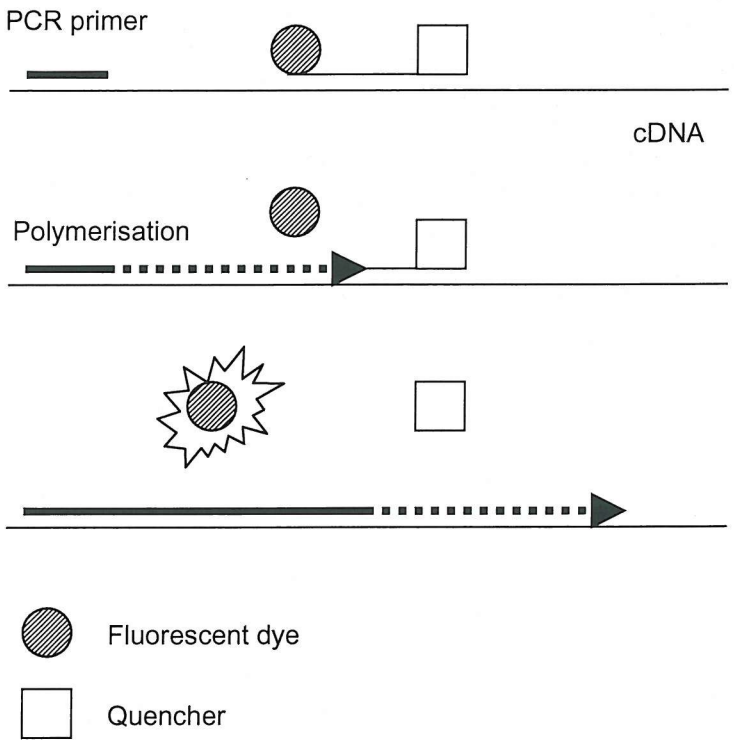


Figure A4
Principles of TaqMan RT-PCR

In samples where the target gene is expressed at greater levels, the fluorescent threshold is detected after fewer cycles of PCR.

Appendix 5

Effect of correcting results to DNA

Certain assays used in the course of this investigation (^{3}H thymidine, ^{3}H proline and MMP-2/TIMP-1 activity assays) required the results to be expressed as a per cell equivalent and this was done by quantifying double stranded DNA at the end of each assay. Equal numbers of cells were used in these assays but by measuring DNA, variations in adhesion of cancer cells to different matrices and potential changes in cell number amongst PSC treated with conditioned medium were accounted for. This meant that the results were specific to the biological variable being measured. The effect of this correction in the ^{3}H proline and MMP-2/TIMP-1 activity assays was indicated in the relevant chapter. The effect in the ^{3}H thymidine assay with respect to the results obtained with collagen type I and IV is shown below.

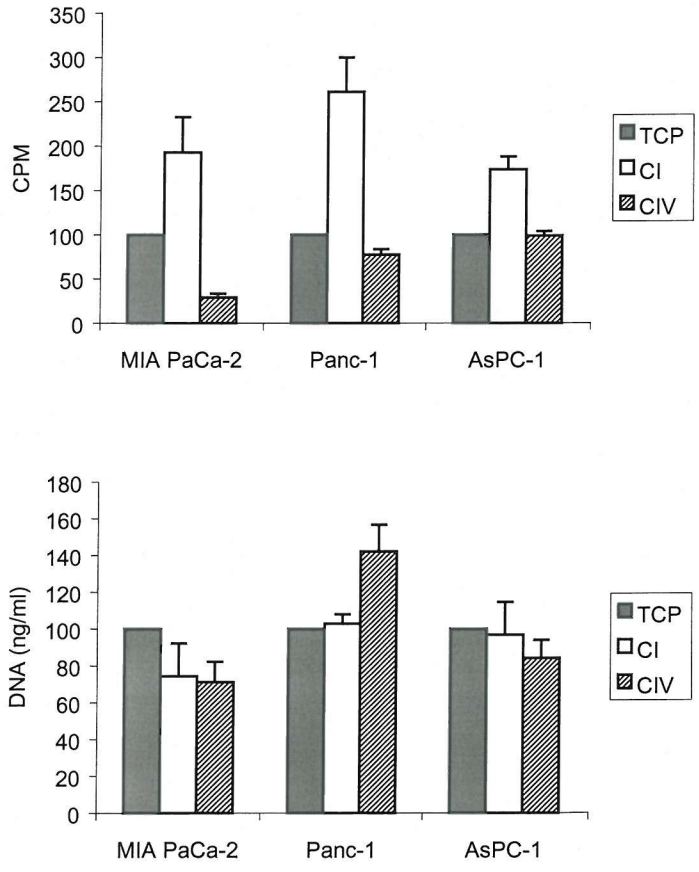


Figure A5
Charts demonstrating the measurement of scintillation and DNA separately in the ^{3}H thymidine assays
Bars represent the mean of 5 or more separate experiments (+/-SEM) and are normalized to results obtained on TCP, which are given an arbitrary value of 100%. The results after correction for DNA were shown in Fig. 5.4

These charts demonstrate that without correction to DNA, the raw scintillation counts give a pattern of results very similar to those presented in Fig. 5.4. MIA PaCa-2 cells did not adhere to collagen type I or IV efficiently and this is reflected by less DNA with respect to TCP at the end of the assay. Conversely the increase in DNA amongst Panc-1 cells cultured on type IV collagen relative to TCP/type I collagen indicated a higher adhesion rate. This particular result made the inhibitory effect of type IV collagen on Panc-1 ^{3}H thymidine incorporation more pronounced. Generally, the overall pattern of ^{3}H thymidine incorporation was not significantly altered by correcting results to DNA.