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UNIVERSITY OF SOUTHAMPTON

AN INVESTIGATION OF THE EFFECT OF TISSUE INHIBITORS OF METALLOPROTEINASES –1 AND –2 ON HEPATIC STELLATE CELL SURVIVAL

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ABSTRACT

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AN INVESTIGATION OF THE EFFECTS OF TISSUE INHIBITORS OF METALLOPROTEINASE-1 AND –2 ON HEPATIC STELLATE CELL SURVIVAL

By Dr. Francis Robert Murphy

During hepatic injury, the hepatic stellate cells (HSC) become activated to a myofibroblast-like phenotype, proliferate and are known to be the major source of matrix proteins which characterise liver fibrosis and cirrhosis. Activated HSC also express matrix degrading metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). Previous work has demonstrated that during spontaneous recovery from experimental liver fibrosis after 4 weeks of carbon tetrachloride injections, there is a fall in the expression of TIMP-1, a loss of the HSCs by apoptosis, and an increase in hepatic collagenolytic activity with the return of the liver to a near normal histology. This correlation between HSC survival and expression of TIMP-1 in vivo highlighted potential roles for TIMP-1 and TIMP-2 affecting HSC survival and apoptosis which was the subject of this study.

The aim of this study was to determine the effect of TIMP-1 and TIMP-2 on HSC survival and determine whether this effect was through effects on inhibition of MMP activity. In vivo studies of experimental liver fibrosis demonstrated a correlation between resolution of fibrosis, falling TIMP-1 mRNA, loss of HSC and transient increase in collagenolytic activity. This data suggested that TIMP-1 was likely to have an effect promoting HSC survival. In vitro studies of culture activated HSC demonstrated that neither TIMP-1 nor TIMP-2 regulated HSC proliferation suggesting that the pro-survival effect was likely to be via inhibition of apoptosis. Incubation with TIMP-1 or TIMP-2 significantly reduced apoptosis of HSC induced by a number of stimuli in a dose-dependent manner. Neutralising antibodies to TIMP-1 and TIMP-2 increased HSC apoptosis compared non-immune IgG control. Whilst both a synthetic selective inhibitor of MMP-2 and a broad spectrum synthetic MMP inhibitor reduced HSC apoptosis, a non-functional mutated TIMP-1 (T2G mutant) had no effect indicating that the inhibition of apoptosis by TIMP-1 was via inhibition of MMP activity. MMP-2 like TIMP-1 is expressed by activated HSC. Addition of recombinant active MMP-2 to HSC resulted in significantly enhanced apoptosis and was associated with cleavage of N-cadherin which could be reduced by co-incubation with recombinant TIMP-1 but not by the non-functional T2G mutant TIMP-1. Furthermore, selective MMP-2 inhibitors protected N-cadherin from cleavage. Active MMP-2 cleaves intact N-cadherin into similar sized fragments in vitro. Blockade of N-cadherin binding with blocking antibody or HAVDI blocking peptide promoted HSC apoptosis, suggesting N-cadherin is a potential target mechanism for the regulation of survival and apoptosis of HSC via active MMP-2 mediated cleavage. Finally, to determine if effects on apoptosis by TIMP-1 were through changes in cell-matrix interactions, HSC plated onto a mutated collagen that is resistant to degradation by MMP-2 were more resistant to apoptosis induced by active MMP-2 than HSCs plated onto normal wild type collagen-1.

In conclusion TIMP-1 regulates HSC apoptosis via MMP inhibition by influencing the MMP mediated degradation of at least two pericellular substrates namely type I collagen and N-cadherin. Together these data demonstrate a novel role for TIMP-1 promoting HSC survival in hepatic fibrosis via stabilising cell-matrix and cell-cell survival signals.

Publications from this thesis

Murphy FR, Issa R, Zhou X, Ratnarajah S, Nagase H, Arthur MJP, Benyon C, Iredale JP (2002) "Inhibition of apoptosis of activated hepatic stellate cells by tissue inhibitor of metalloproteinase-1 is mediated via effects on matrix metalloproteinase inhibition: Implications for reversibility of liver fibrosis" Journal of Biological Chemistry, vol 277, No13, March 29 11069-11076.

Murphy FR and Iredale JP. "Resolution of fibrosis by apoptosis of myofibroblasts." Book chapter 15. Cytokines in Liver injury and repair - FALK symposium 125. Kluwer academic publishers.

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Murphy FR, Issa R, Hussain H, Ratnarajah S, Soloway P, Nagase H, Arthur MJP, Benyon C, Iredale JP " Inhibition of apoptosis of activated hepatic cells by TIMP-1 is mediated via effects on MMP inhibition: Implications for reversibility of liver fibrosis" Oral presentation at British Society of Gastroenterology, March 2002

Murphy FR, Wuang J, Collins J, Arthur MJP, Nagase H, Mann D, Benyon RC, Iredale JP "N-Cadherin cleavage during activated hepatic stellate cell apoptosis is inhibited by Tissue inhibitor of metalloproteinase-1" Oral presentation at the British Association of the Study of Liver, September 2002

Murphy FR, Zhou X, Issa R, Hussain H, Waung J, Patel N, Collins J, Brew K, Nagase H, Arthur MJP, Benyon RC, Iredale JP."The balance of TIMPs and MMP-2 determines hepatic stellate cell fate during recovery from liver fibrosis" Oral presentation at the Medical Research Society as Young Investigator Award finalist, Royal College of Physicians London, February 5th 2003

Murphy FR, Waung J, Patel N, Collins J, Brew K, Nagase H, Arthur MJP, Benyon RC, Iredale JP. "Balance of TIMP-1 and MMP-2 determines N-Cadherin cleavage in hepatic stellate cell apoptosis" Oral presentation at the British Society of Gastroenterology, March 2003

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International presentations from this thesis

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Murphy FR, Issa R, Hussain H, Ratnarajah S, Soloway P, Nagase H, Arthur MJP, Benyon C, Iredale JP " Inhibition of apoptosis of activated hepatic cells by TIMP-1 is mediated via effects on MMP inhibition: Implications for reversibility of liver fibrosis" Poster presentation at Digestive Diseases Week, May 2002, San Francisco, USA.

Murphy FR, Wuang J, Collins J, Arthur MJP, Nagase H, Mann D, Benyon RC, Iredale JP "N-Cadherin cleavage during activated hepatic stellate cell apoptosis is inhibited by Tissue inhibitor of metalloproteinase-1" Oral presentation at the 52nd International symposium of the cells of the hepatic sinusoid meeting, August 2002, Tucson, Arizona, USA.

Murphy FR, Wuang J, Collins J, Arthur MJP, Nagase H, Mann D, Benyon RC, Iredale JP "N-Cadherin cleavage during activated hepatic stellate cell apoptosis is inhibited by Tissue inhibitor of metalloproteinase-1" Poster presentation at the American Association for the study of Liver Diseases, November 2002, Boston, USA.

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LIST OF ABBREVIATIONS

APAF-1	Apoptotic protease activating factor
AP-1	Activator protein –1
APMA	p-aminophenylmercuric acetate
Bak	BCL-2 antagonist/killer 1
Bax	BCL-2 associated X protein
BLK	B lymphoid tyrosine kinase
Bcl-2	B cell lymphoma-2
C_T	Cycle threshold
DEPC	Diethylpyrocarbonate
DDR	Discoidin domain receptor
ECM	Extracellular matrix
ET-1	Endothelin-1
ERK	Extracellular signal related kinase
ESB	Electrophoresis sample buffer
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HBSS	Hank's buffered salt solution
HSC	Hepatic stellate cell
IAP	Inhibitor of apoptosis protein
IGF-1	Insulin like growth factor-1
MAPK	Mitogen activated protein kinase
MCL-1	Myeloid cell leukaemia sequence-1
MMP	Matrix metalloproteinase
MOPS	Morpholinopropanosulfonic acid
NF κB	Nuclear factor kappa B
NGF	Nerve growth factor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PI3	Phosphatidyl inositol 3 phosphate
SAMe	S-adenosyl-L-methionine
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SF	Cells cultured in serum free media

STAT	Signal transducer and activator of transcription
TGFβ	Transforming growth factor beta
TIMP	Tissue inhibitors of metalloproteinase
TNFα	Tumour necrosis factor alpha
TRAF	TNF receptor associated factor
TUNEL	Terminal dUTP nick end labelling
UPA	Urokinase plasminogen activator

Chapter 1

1.1 Liver fibrosis: The scale of disease.

1.1.1 Liver fibrosis and its end stage, cirrhosis, present a major world-wide health problem. The World Health Organisation estimates that 3% (170 million) of the world population are infected with hepatitis C virus alone. Hepatic fibrosis represents a common pathway for many types of chronic liver injury including alcoholic damage, viral infection with hepatitis B and C and immune mediated damage (Alcolado et al. 1997). It can be viewed as an end point of the inflammation repair resolution continuum, where the mechanism of resolution is either inhibited or overwhelmed by repetitive injury. At present, the only effective therapy for end stage disease is liver transplantation. In practice, this treatment is appropriate for only a minority of highly selected patients. This leaves many patients with significant liver disease and poor prognosis. Effective treatment for some specific forms of liver fibrosis is available. These include removing the liver injury itself (for example alcohol in alcoholic liver disease) and specific therapeutic interventions. Examples include anti viral therapy for hepatitis B (Schiff et al. 2000) and hepatitis C virus infection (Poynard et al. 2000, Dusheiko and Roberts 1995), steroid therapy for autoimmune hepatitis (Dufour et al. 1997) and venesection for hereditary haemochromatosis (Powell et al. 1980). These treatments are most effective if started early in the natural history of these diseases, however a significant number do not respond or are intolerant to the treatment for clinical reasons. The lack of current treatments to directly reduce liver fibrosis or indeed fibrotic disease of other solid organs has lead to a great expansion of basic science research into the myofibroblast-like activated hepatic stellate cell.

1.1.2 The starting point of this work is that the activated hepatic stellate cell is accepted to play an important role in promoting liver fibrosis because it is the major source of matrix proteins in liver fibrosis (described below) (Maher and McGuire 1990). Furthermore, these cells inhibit matrix degradation by secreting the potent tissue inhibitors of metalloproteinase – 1 and –2 (TIMP-1 and TIMP-2) (Iredale et al. 1992). There is good evidence (Milani et al. 1992a; Herbst et al. 1991; Vyas et al. 1995b; Winwood et al. 1995; Arthur et al. 1989; Iredale et al. 1996; Benyon et al. 1996) that matrix degradation is inhibited in progressive liver fibrosis therefore favouring matrix accumulation. It is the hope of many physicians around the

world today that this type of research will eventually lead to novel therapies for liver fibrosis and cirrhosis in the future.

1.2 The evidence that demonstrates the activated HSC as the source of ECM in the fibrotic liver

1.2.1 It is important to understand the background data that have led to the conclusion that the activated HSC is the source of ECM in the fibrotic liver. Many research groups world wide have focused on the activated HSC as a target for anti fibrotic therapy. The conclusions may be summarised as below:

1. In vitro studies from the early 1980s demonstrated that the collagen found in cultures of primary hepatocytes was due to contaminating HSCs within the cell monolayers (Maher et al. 1988). Further studies confirmed that HSCs synthesise large amounts of collagen while other cell types (e.g. hepatocytes, sinusoidal endothelial cells) make relatively little (Friedman et al. 1985; Matsuoka and Tsukamoto 1990).
2. In vivo studies using immunocytochemistry and in situ hybridisation demonstrated that collagen expression in fibrotic livers was present in activated HSC rather than parenchymal cells (Milani et al. 1990).
3. In vitro studies of extracted rat HSC have demonstrated that while quiescent HSC express low levels of mRNA for procollagen I and III, activated HSC express high levels (Stefanovic et al. 1997).
4. HSCs extracted from fibrotic livers demonstrate over expression of ECM genes which is not observed in other hepatic cell types (Milani et al. 1995).
5. Studies of both experimental and human examples of liver fibrosis have demonstrated a positive correlation between the degree of fibrosis and the accumulation of activated HSC in the injured liver (Knittel et al. 1999; Krane et al. 1996).
6. Studies of experimental liver fibrosis have demonstrated that during spontaneous resolution of liver fibrosis loss of activated HSC by apoptosis or programmed cell death is accompanied by a loss of fibrotic tissue with return of the liver to a near normal histological appearance (Iredale et al. 1997; Issa et al. 2001).
7. Agents that reduce activation or proliferation of HSCs also reduce progression of hepatic fibrosis in experimental models of chronic liver injury (Baroni et al. 1996; Pines et al. 1997)

8. Agents that promote apoptosis of activated HSC also promote resolution of hepatic fibrosis (Wright et al. 2001).

1.2.2 Given these basic observations about HSC biology it is not surprising that many groups have focused on HSC biology to gain a wider understanding of the pathogenesis of liver fibrosis and cirrhosis. In particular, greater understanding of the process mediating spontaneous recovery of liver fibrosis will define the attributes of an effective anti fibrotic therapy. The overall intent being the rational design of novel anti fibrotic therapies that are suitable for use in any form of liver fibrosis, that is to say a generic anti fibrotic drug. There are many promising approaches for new treatments for liver fibrosis and they are described in detail later.

1.3 Microanatomy of the hepatic sinusoid in normal and fibrotic liver

1.3.1 In the normal healthy liver the hepatic stellate cells (HSC) exist in the space of Disse. This space is between the fenestrated sinusoidal endothelial cells and the hepatocyte brush border (Figure 1.1). The space of Disse contains a fine basement membrane like matrix consisting of type IV collagen, laminin and proteoglycan. The quiescent HSC are rich in retinoids and lipid droplets. As a result of liver injury, the HSC undergo a phenotypic change and proliferate to become myofibroblast-like cells (described in detail below). In this so-called "activated" phenotype, HSCs are the major source of the matrix that accumulates in fibrosis. In addition to the overall increase in extracellular matrix that accumulates in the space of Disse in fibrosis, qualitative changes also occur, in that fibrillar collagens type I and III now predominate. As fibrosis progresses there is architectural disturbance of the liver parenchyma. Ultimately, collagenous bands link vascular structures with resulting disruption to hepatic blood flow and parenchymal function (Friedman 1993). This is associated with the development of portal hypertension, hepatic decompensation and the clinical sequelae that are apparent in the clinic (described below).

FIGURE 1.1 COMPARISON OF MICROANATOMY OF NORMAL VERSUS FIBROTIC LIVER

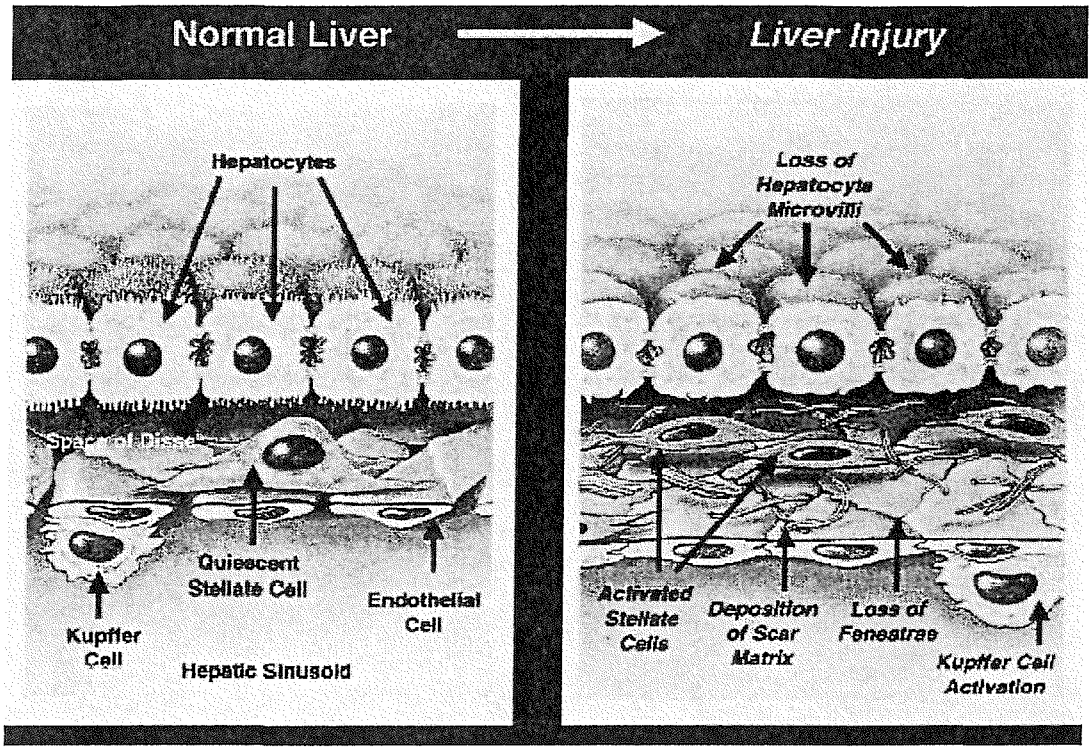


Fig. 1.1 Changes in the sinusoid during fibrotic liver injury. The changes that occur in the subepithelial space of Disse include changes in cellular and matrix components. Stellate cell activation to a fibrogenic phenotype leads to accumulation of scar tissue (predominantly fibrillar collagens). This change in extracellular matrix contributes to alterations in other cell types including the loss of hepatocyte microvilli and endothelial fenestrae. Overall the change from a low density to higher density matrix in the space of Disse and the loss of hepatocyte villi and endothelial fenestrae compromise the flow of molecules to the hepatocyte causing deterioration in liver functions in toto. Figure taken from (Friedman 2000).

1.4 Clinical effects of liver cirrhosis

1.4.1 The clinical features of liver disease are legion. It is beyond the scope of this thesis to give a comprehensive description of the clinical effects of liver disease. However, there is a spectrum of disease with mild liver fibrosis causing little if any problems for the patients while those with severe fibrosis and architectural disturbance (liver cirrhosis) suffer numerous problems affecting every physiological system of the body. This results in a shortened life span and poor quality of life for such patients. At the present time, the scope for effective therapy for such patients excluding liver transplantation is limited. As a consequence of this, many patients experience the natural history of advanced liver disease dying eventually of a

combination of pathological consequences of liver disease such as gastrointestinal haemorrhage, hepatic encephalopathy, ascites and spontaneous bacterial peritonitis, renal failure and other medical problems.

1.5 Current therapy for liver fibrosis: remove the causal agent

1.5.1 As simple as it might seem, removal of the causal agent is currently the best treatment available for many types of liver disease. This is most effective when instituted in the early stages of hepatic fibrosis with the potential resolution of the liver to near normal histology. This approach is effective in iron overload (Hereditary Haemochromatosis), copper overload (Wilson's disease), excessive alcoholic consumption, chronic hepatitis B virus and hepatitis C virus infection (Poynard et al. 2000; Davis et al. 1998), autoimmune chronic active hepatitis, schistosomiasis, secondary biliary fibrosis and drug induced liver disease (Ramm et al. 1997; Schilsky et al. 1991; Shiratori et al. 2000 ; Dufour et al. 1997; Emonard and Grimaud 1989; Hammel et al. 2001).

1.5.2 Chronic hepatitis C virus infection is now the most common cause of cirrhosis in Western countries and represents a major world-wide healthcare burden. The prevalence of chronic hepatitis C virus infection in the UK has been estimated to be between 0.08% and 0.8% depending on the risk groups studied (Lodi et al. 1997; Ward et al. 2000). It has been found to be as high as 70% in high risk groups such as intravenous drug users(Taylor et al. 2000). Unfortunately, conventional treatment with Interferon alpha and Ribavirin is at best successful in about half of all cases. Furthermore, many patients are not suitable for this therapy for clinical reasons (for example concurrent depression) and therefore endure the natural history of this disease. The natural history of patients infected with hepatitis C virus is a slow progression of liver fibrosis with all its clinical consequences. Progression is fastest in male alcoholic patients who have acquired the infection over the age of 40 years of age (Poynard et al. 1997). Given the considerable current and expected future clinical need for anti fibrotic therapy for patients with chronic hepaititis C virus alone, much effort has been directed at the HSC as a target of therapeutic intervention.

1.6 Hepatic stellate cells and liver fibrosis

1.6.1 Hepatic stellate cells are recognised as central players in the fibrotic process. The HSC in the normal liver exist in a quiescent state and contain vitamin A in the form of retinylesters.

Following injury they undergo a dramatic phenotypic change to myofibroblast-like activated HSCs (Figure 1.2). These activated HSC express a number of cytoskeletal markers and characteristics of smooth muscle and fibroblast cells (Friedman 1993). As a part of the phenotypic transformation the activated HSC proliferate, demonstrate contractile and migratory properties, promote fibrogenesis, increase matrix turnover, and promote chemotaxis of white blood cells including monocytes or Kupffer cells (Friedman 2000).

1.6.2 Research into the function of HSC has been facilitated by the observation that cells extracted from normal rat or human liver and plated onto uncoated tissue culture plastic, undergo a phenotypic activation in a manner that mimics the process in vivo. Using this model and in vivo models of liver fibrosis, activated HSCs have been demonstrated to be the major source of matrix proteins (type I and III collagen and others) in fibrotic liver (Friedman et al. 1985). In generic terms the activation of the HSC can be viewed as part of the liver wound healing response and reflects this response in other tissues including the wound healing in the skin (Clark 1993) and experimental glomerulonephritis (Mizuno-Horikawa et al. 2001).

1.7 Mechanisms of hepatic stellate cell activation

1.7.1 There are a number of recognised mechanisms of HSC activation. Firstly, activation may occur via activated Kupffer cells, which release a series of soluble factors that directly act on the HSC to promote its activation and proliferation. These include platelet-derived growth factor, transforming growth factor beta-1 and novel low molecular weight factors (Arthur et al. 1989; Armendariz-Borunda et al. 1989; Shiratori et al. 1986). Secondly, damaged hepatocytes or those undergoing apoptosis release soluble factors, which promote proliferation of HSCs. These include insulin-like growth factor-1, fibroblast growth factor and transforming growth factor-α (Gressner et al. 1992). Thirdly, oxidant stress is also recognised to activate HSCs through the NF-kappaB and c-myb transcription factor pathways (Lee et al. 1995).

1.8 Mechanisms of maintaining the activated HSC phenotype: Paracrine and autocrine cytokine activity and ECM remodelling.

1.8.1 Once activated, the HSC proliferate and participate in a number of autocrine and paracrine pathways that maintain their activation (Figure 1.2). These factors include a wide array of pro-inflammatory and pro-fibrogenic cytokines, growth factors and chemokines. Of particular importance is the autocrine secretion of transforming growth factor-β1 which upregulates collagen-I expression and inhibits the collagen degradative pathways (Bachem et al. 1992; Friedman and Yamasaki 1991). In addition to increases in TGF-β, TGF-β receptor expression is increased also. Indeed, the general family of tyrosine kinase receptors, which mediate many of the HSC response to cytokines, is generally up-regulated during liver injury (Ankoma-Sey et al. 1998). The combined effect of these growth factors on HSCs is the increased synthesis of matrix proteins with inhibition of matrix degradation by expression of TIMP-1 and TIMP-2 that reduce net collagenolytic activity.

1.8.2 The increase ECM remodelling gradually occurs with a change from a low density matrix to a higher density matrix containing fibrillar collagens. This change in matrix affects not only the HSC but also endothelial cells and hepatocytes (Figure 1.1). The new matrix interactions further stimulate stellate cell activation. This is thought to be mediated by at least two classes of molecules. Firstly, integrins which are the classical ECM receptors and secondly a novel member of the tyrosine kinase receptor family known as the discoidin domain receptor (DDR). A number integrins have been found in activated HSC including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_V\beta_1$, $\alpha_V\beta_3$, and $\alpha_6\beta_4$ (Pinzani et al. 1998). DDR-2 has been shown in HSC (Ankoma-Sey et al. 1998) and is a novel tyrosine kinase receptor whose ligand is fibrillar collagen rather than soluble growth factor (Shrivastava et al. 1997, Vogel et al. 1997). Together the cell-matrix interaction between HSC and the new matrix (especially type I collagen) promotes perpetuation of the active HSC phenotype.

FIGURE 1.2 PHENOTYPIC FEATURES OF HEPATIC STELLATE CELL ACTIVATION DURING LIVER INJURY AND RESOLUTION

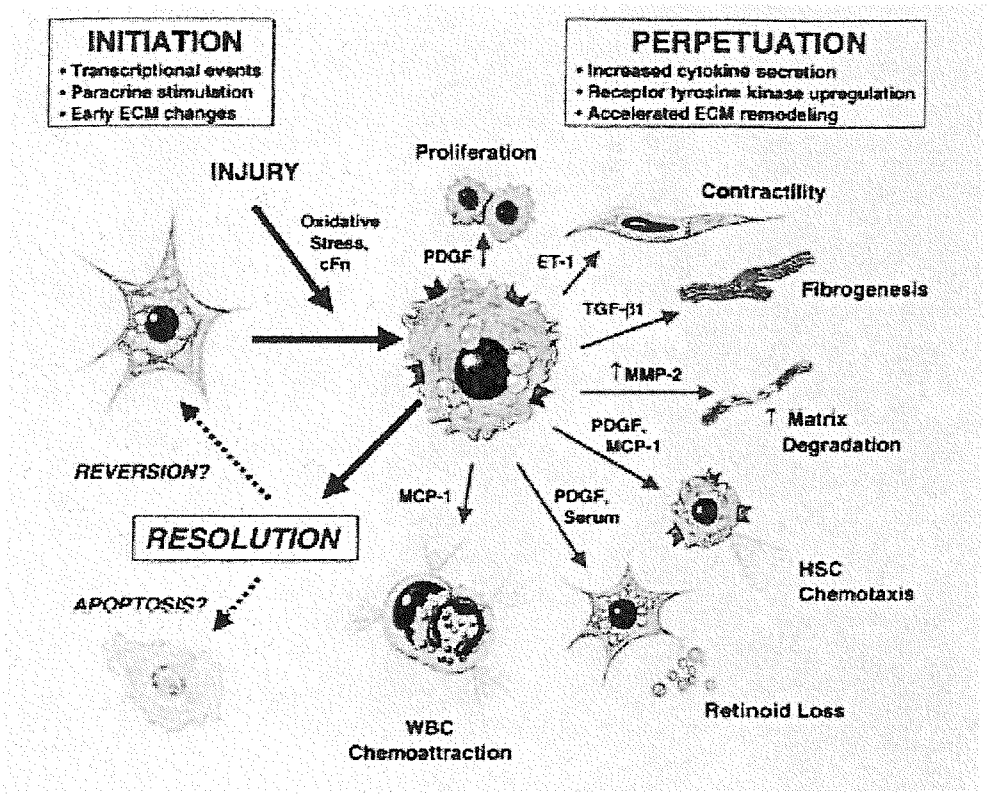


Fig.1.2. Phenotypic features of hepatic stellate activation during liver injury and resolution. After liver injury quiescent HSC undergo a phenotypic transformation termed 'activation' which encompasses the change to a myofibroblast-like phenotype. Reproduced from (Friedman 2000)

1.9 Study of the hepatic stellate cell in liver fibrosis

1.9.1 Research into the pathogenesis of liver fibrosis has been facilitated by the observation that HSC are the major mediators of the fibrotic process. Moreover, in vitro culture of extracted hepatic stellate cells and animal models have provided invaluable insights into hepatic fibrogenesis. Animal models include the carbon tetrachloride model of liver fibrosis (Iredale et al. 1998) and the bile duct ligation model in the rat (Issa et al. 2001). In both models liver fibrosis may be induced in a reproducible manner. Furthermore, resolution of fibrosis may be observed to occur when the liver injury is stopped (cessation of injection or biliary jejunal anastomosis).

1.9.2 These models have allowed identification of the activated hepatic stellate cell as the key cell type responsible for production of the extracellular matrix in chronic liver disease. Furthermore, they have highlighted that the stellate cell interacts with many other cell types within the liver including hepatocytes, sinusoidal endothelial cells, lymphocytes, neutrophils and Kupffer cells (liver macrophages). Many of these interactions play an important role in stellate cell biology and have been exploited to design and evaluate novel strategies and potential anti-fibrotic therapies (described below). Indeed, understanding the detailed molecular biology of the HSC will allow the understanding of the multiple potential therapeutic targets that exist within the pathobiology of liver fibrosis.

1.10 The current knowledge of potential therapeutic approaches for liver fibrosis and their limitations.

1.10.1 The HSC cell has been recognised to be responsible for synthesis of much of the excess extracellular matrix observed in chronic liver diseases. In its quiescent state, the HSC functions mainly as the site of storage of vitamin A as retinyl esters. Following liver injury the quiescent HSC undergo a phenotypic change into a myofibroblast-like cell. During this process (termed "activation") cells shed their vitamin A droplets and begin to proliferate, migrate, recruit inflammatory cells, become contractile and synthesise fibrillar collagen. In addition to synthesis of new collagen, the activated stellate cells make matrix metalloproteinases (MMPs) and their specific inhibitors: tissue inhibitors of metalloproteinase (TIMPs). MMPs are a family of 28 calcium dependent proteases that are able to degrade a large number of extracellular matrix components including fibrillar collagen (see later). If the underlying cause of liver injury is removed, there is resolution of the inflammation and remodelling of extracellular matrix with resolution of liver histology to near normal appearance. This pattern of spontaneous resolution of liver fibrosis has been observed in two animal models, carbon tetrachloride intoxication and bile duct ligation(Iredale et al. 1998; Issa et al. 2001). One theme that has emerged is that the population of activated HSC undergo apoptosis during spontaneous resolution of liver fibrosis. This has stimulated research into the factors regulating HSC activation, proliferation, survival and apoptosis.

These detailed observations about HSC pathophysiology have the potential to allow the rational design of novel classes of anti-fibrotic therapies as follows:

1. Agents directed to reduce hepatic injury
2. Inhibitors of HSC activation.
3. Antioxidants.
4. Agents blocking HSC proliferation.
5. Agents blocking intracellular signalling.
6. Agents blocking HSC differentiation.
7. Agents blocking cell matrix interactions.
8. Agents to promote HSC apoptosis.
9. Agents directed to reduce collagen synthesis.
10. Agents directed to promote collagen degradation.

These subdivisions are merely to help classify the potential therapeutic mechanisms that have been elucidated so far. It is important however to appreciate that some agents act through more than one mechanism (for example TGF-β inhibition described later).

1.10.2 Agents directed to reduce hepatic injury

1.10.2.1 Inflammation within the liver has been shown to promote liver fibrosis. Indeed, in human liver disease there is a correlation between hepatic inflammation and progression of liver fibrosis (McCaughan et al. 2000). The cellular inflammatory infiltrate observed during some types of liver fibrosis has been shown to promote collagen synthesis in hepatic stellate cells through an oxidative stress mechanism (Casini et al. 1997). These observations have led to the evaluation of a number of anti inflammatory agents.

1.10.2.2 Corticosteroids have been used for many years to successfully treat autoimmune chronic active hepatitis. However, corticosteroids have multiple undesirable side effects and so their clinical use has been confined mainly to this type of liver fibrosis. It is not clear if corticosteroids have a direct anti fibrotic effect (Niki et al. 1996). For patients with autoimmune hepatitis, corticosteroids slow progression from fibrosis to cirrhosis. In addition, there are case reports of patients in whom resolution of fibrosis to near normal histology has occurred with corticosteroid therapy (Dufour et al. 1997). Azathioprine has been used as a steroid sparing agent and this is a useful way of limiting the long-term side effects of corticosteroids. Corticosteroid therapy appears to reduce short term mortality for patients with severe acute alcoholic hepatitis (Mathurin et al. 1996). Randomised clinical trials of Corticosteroid therapy have not shown benefit in patients with Primary Biliary Cirrhosis (Mitchison et al. 1992) or Primary Sclerosing Cholangitis (Lindor et al. 1991).

1.10.2.3 Colchicine is well known to have anti-inflammatory effects. It has been used for the treatment of liver fibrosis on the basis of a study that showed improved survival in cirrhotic patients (Kershenobich et al. 1988). Controversially, further studies into the efficacy of Colchicine have proved to be not effective in improving survival or need for transplantation in patients with Primary Biliary Cirrhosis (Poupon et al. 1996).

1.10.2.4 One group of therapies in development include those directed to neutralise pro inflammatory cytokines for example tumour necrosis factor alpha (TNFα). Infliximab, a

genetically engineered monoclonal antibody to neutralise human TNFα has been used successfully to treat Crohn's disease (a type of inflammatory bowel disease) in humans (Present et al. 1999). It is thought that TNFα is important in the pathogenesis of severe acute alcoholic hepatitis and a clinical trial is currently progress to evaluate its efficacy in this group (Jalan et al. 2001). Thalidomide also behaves as a TNFα antagonist and has been shown to be beneficial in experimental models of liver fibrosis (Muriel et al. 2001). Soluble TNFα receptors and interleukin-1 receptor antagonists have also been shown to reduce necrosis and inflammation in experimental animal models of liver fibrosis (Bruck et al. 1997; Mancini et al. 1994).

1.10.2.5 Interleukin-10 (IL-10) is known to have potent anti inflammatory and anti fibrotic effects. IL-10 (-/-) knockout mice develop a more severe liver fibrosis than wild type mice suggesting a possible role for this cytokine as a therapy (Louis et al. 1998). IL-10 has been shown to reduce fibrosis in a pilot study in patients infected with hepatitis C virus that have not responded to conventional therapy (Nelson et al. 2000). The results of larger trials are awaited.

1.10.3 Inhibitors of HSC activation.

1.10.3.1 The activation of stellate cells has been shown to involve a number of key mechanisms. Firstly, paracrine stimuli from injured cells (including hepatocytes, Kupffer cells, leukocytes, platelets, sinusoidal endothelial cells) directly prime quiescent hepatic stellate cells to activate(Friedman and Arthur 1989; Maher 1999; Gressner et al. 1995). Secondly, damaged hepatocytes and Kupffer cells produce lipid peroxides leading to oxidative stress and stellate cell activation. Agents that reduce oxidative stress have been shown to reduce liver fibrosis (see below). Thirdly, a number of key cytokines can activate stellate cells. These include TGFβ, platelet-derived growth factor (PDGF) and endothelin-1 (ET-1). The downstream intracellular signalling pathways for these cytokines have been studied in detail and include transcription factors including nuclear factor κB, c-myb, c-jun, AP-1, STAT-1 and SMAD proteins (Reimann et al. 1997; Pinzani et al. 1998; Marra et al. 1999).

1.10.3.2 Following liver injury the HSC enter the cell cycle and become activated. Transcription factors controlling growth and differentiation change, also histone proteins

become deacetylated by histone deacetylase (Niki et al. 1999). The peroxisome proliferation activated receptors (PPAR) control growth and differentiation in a number of tissues. During activation of HSC PPAR-γ levels decrease. PPAR-γ ligands reduce fibrotic and inflammatory actions of activated HSC (Miyahara et al. 2000; Marra et al. 2000). Once activated, HSC maintain their activated phenotype by a number of autocrine loops including synthesis of TGFβ and its receptors (Friedman et al. 1994), type 1 collagen and its integrin receptors (Sato et al. 1998). Activated HSC also prevent collagen degradation by autocrine production of TIMP-1. The downstream pathways of the cytokines that promote stellate cell proliferation include ras/ERK/MAP kinase, PI3-kinase and STAT-1 (Pinzani et al. 1998). The pro proliferative effect of PDGF involves calcium ion influx and is sensitive to intracellular pH and Na/H exchanger activity (Failli et al. 1995; Di Sario et al. 1999).

1.10.3.3 Given this detailed understanding of the molecular biology involved in stellate cell activation and proliferation, several therapeutic agents have targeted these mechanisms. These include antioxidants, agents blocking cell proliferation, agents blocking intracellular signalling and agents blocking cell growth and differentiation and others described below.

1.10.4 Antioxidants

1.10.4.1 Given the key role of oxidative stress in the activation of hepatic stellate cells, many antioxidant substances have been evaluated as potential antifibrotic drugs. Vitamin E (α-tocopherol) has been shown to reduce hepatic stellate cell activation by reducing lipid peroxidation. It has been tested in experimental models of liver fibrosis and appears to reduce fibrogenesis in carbon tetrachloride induced fibrosis (Parola et al. 1992). This beneficial effect was not observed in a human clinical trial examining the effect of long term Vitamin E administration in alcoholic cirrhotics (de la Maza et al. 1995). Glycyrrhizin is a polysaccharide extract from liquorice has antioxidant properties and has been shown to reduce fibrosis in the rat bile duct ligation model of liver fibrosis (Park et al. 1997). Resveratrol is a natural extract of grapevine with antioxidant properties. It has been claimed to deactivate activated stellate cells (Godichaud et al. 2000). Phosphatidylcholine (PPC) is a polyunsaturated phospholipid extract from soy beans. It has been evaluated in patients with alcoholic disease on the basis of earlier studies that showed it had antioxidant and anti-fibrogenic properties in alcoholic injury in animals (Aleynik et al. 1997; Ma et al. 1996). It has been shown to reduce activated HSC numbers in alcohol induced liver injury (Poniachik

et al. 1999). The results of a multicenter trial of PPC of patients with alcoholic liver disease are awaited.

1.10.4.2 One interesting observation of quiescent HSC is that they store vitamin A as retinyl esters. Retinyl palmitate has antioxidant properties and can prevent experimental liver fibrosis induced by dimethylnitrosamine or pig serum (Mizobuchi et al. 1998). Hepatic glutathione has been shown to have hepatoprotective and antioxidant roles. Substrates for glutathione synthesis have been used traditionally in a number of human liver diseases including acute paracetamol overdose, alcoholic liver disease, drug induced liver disease and others. Oral methionine, intravenous N-acetyl-cysteine and oral S-adenosyl-L-methionine (SAMe) all promote glutathione synthesis. The effect of 1200mg/day of oral SAMe in patients with alcoholic liver cirrhosis was recently the subject of a randomised placebo controlled trial. SAMe treatment appeared to significantly benefit patients who had less advanced liver disease while there was no significant benefit for those patients with the most advanced liver cirrhosis in terms of mortality and need for liver transplantation (Mato et al. 1999).

1.10.4.3 Dietary intake of fatty acids have been shown to have an impact of the development of liver fibrosis in experimental models. While diets containing excessive polyunsaturated fatty acids enhance experimental liver fibrosis (Fernandez et al. 1997), diets containing excess saturated fatty acids have been shown to reverse fibrosis in alcohol induced liver fibrosis in rats (Nanji et al. 1997).Whether these observations translate into effects relevant in human liver disease remains to be seen.

1.10.4.4 Silymarin is a flavinoid antioxidant extract from Silybum marianum with anti fibrotic effects (Boigk et al. 1997). It has recently been the subject of a randomised placebo controlled clinical trial of patients with alcoholic liver disease. No significant benefit was observed in patients that received 450mg/day of silymarin orally over those that received placebo alone (Pares et al. 1998).

1.10.4.5 Overall, antioxidant approach to treat liver fibrosis has certain advantages mainly that these agents do not appear to have major adverse side effects while a disadvantage with these agents is that they need to be taken on a long term basis to maximise their therapeutic effect. This introduces other problems for example compliance with treatment.

1.10.5 Agents blocking proliferation

1.10.5.1 Following liver injury, HSC proliferate and therefore much effort has focused on agents that can prevent HSC proliferation. PDGF is known to be a potent mitogen for activated hepatic stellate cells. Its action is sensitive to intracellular pH and involves calcium influx in stellate cells. PDGF mitogenic response can be prevented by treatment with Amiloride which interferes with Na/H exchanger activity (Di Sario et al. 1999) or calcium channel antagonists including diltiazem, nifedipine and verapamil (Kataoka et al. 1997). Rapamycin inhibits proliferation of HSC and has demonstrated an anti-fibrotic effect in experimental models of liver fibrosis (Zhu et al. 1999). These agents targeting PDGF are yet to be studied in human liver fibrosis.

1.10.5.2 Vasoconstrictive agents are known to have mitogenic effects on hepatic stellate cells. Thrombin(Marra et al. 1995) and Angiotensin-II (Bataller et al. 2000) have been shown to promote stellate hepatic proliferation. A number of vasodilator agents including the Angiotensin converting enzyme inhibitor captopril(Jonsson et al. 2001) and nitric oxide donors(Failli et al. 2000) inhibit proliferation of hepatic stellate cells and have shown anti-fibrotic effects in some experimental models of liver fibrosis.

1.10.6 Agents blocking intracellular signalling

1.10.6.1 As previously described, PDGF is a potent mitogen for hepatic stellate cells. The PDGF intracellular signalling pathway is via ERK (extracellular signal related kinase) and H-ras. This signalling may be prevented by exposure to Pentoxifylline (a phosphodiesterase inhibitor). It has been shown to reduce stellate cell proliferation in vitro but its effect on experimental liver fibrosis is not known (Windmeier and Gressner 1997). The H-ras pathway has been shown to be important in hepatic stellate cell proliferation. Inhibition of this pathway by the ras antagonist S-farnesylthiosalicylic acid has been demonstrated to reduce proliferation of hepatic stellate cells and reduced liver fibrosis in thioacetamide-induced liver fibrosis in rats (Reif et al. 1999). In principle, blockade of intracellular signalling of PDGF appears to reduce proliferation of HSC and reduces fibrosis in experimental models. These agents have not yet been evaluated in human liver disease.

1.10.7 Agents blocking growth and differentiation

1.10.7.1 Agents blocking growth and differentiation of HSC exist. The Peroxisome proliferator activated receptors (PPAR) are a family of receptors controlling cell growth and differentiation. PPAR-γ is a transcription factor that decreases during activation of HSC. Ligands for PPAR-γ inhibit the profibrotic and proinflammatory in HSC and promote collagen degradation in the liver (Miyahara et al. 2000).

1.10.7.2 HSC activation includes changes to a proliferative phenotype introducing the notion that agents blocking the cell cycle may be effective in liver fibrosis. The semi-synthetic analogue of fumigillin (TNP-470) is an agent that was developed as an anti cancer agent with anti angiogenic properties. It blocks the cell cycle transition from G1 to S phase and has been shown to prevent HSC activation and reduces fibrosis in experimental models of liver fibrosis (Wang et al. 2000).

1.10.7.3 During HSC activation and differentiation, histone proteins become deacetylated. This has highlighted Trichostatin A (a histone deacetylase inhibitor) for study. This agent reduces HSC activation in vitro (Niki et al. 1999). In principle, these agents prevent HSC differentiation and are potential novel therapies for liver fibrosis.

1.10.7.4 One of the problems with this and other drugs that paralyse critical cell machinery is that their application to an organ specific human disease, such as liver fibrosis, is dependent upon the development of a specific organ targeting system (see later). Without targeted therapy, systemically administered drugs that affect cell proliferation or differentiation are likely to be prone to similar side effects that the anti cancer drugs are well known for (for example bone marrow suppression).

1.10.8 Agents blocking cell matrix interactions

1.10.8.1 The survival of HSC appears to depend on a balance between the pro-survival and pro-apoptotic signals that the cells receive (see later). HSC bind to components of the surrounding extracellular matrix via integrin cell surface receptors and other extracellular matrix receptors. Blockade of cell matrix adhesion at least in part may be achieved using Arg-Gly-Asp peptides (RGD). This has been shown to reduce focal adhesion kinase activity, stellate cell activation and liver fibrosis in experimental models (Bruck et al. 1996; Bruck et al. 1997) however these agents have not been evaluated in human liver disease.

1.10.9 Agents to promote HSC apoptosis

1.10.9.1 Spontaneous resolution of experimental liver fibrosis is accompanied by removal of the activated HSC population by apoptosis (Iredale et al. 1998). This observation has led to greater study into the mechanisms that may promote apoptosis of activated hepatic stellate cells. Apoptosis or programmed cell death is a ubiquitous phenomenon of cell biology. It has been shown to be important during development and has been shown to be involved in many disease processes where there is either too little apoptosis (for example cancer) or too much inappropriate apoptosis (for example neurodegenerative disease). Apoptosis may be initiated by basically one of two mechanisms (Figure 1.9). Firstly, ligand binding to cell surface death receptors produces a cascade of intracellular enzymes known as caspases that are responsible for intracellular disassembly. Secondly, the mitochondria membrane contains a family of proteins all containing the BH-3 motif. Some proteins prevent apoptosis (for example Bcl-2) while others promote the formation of a transmembrane pore that allows cytochrome C to leak into the cytosol (for example bax, bid, bak). This is the point of no return as cytochrome C is rapidly complexed with another protein called APAF-1. This complex rapidly activates the caspase proteolytic cascade.

1.10.9.2 Given this information, HSC are known to express a number of cell surface death receptors including Fas, TNF-α receptors and low affinity nerve growth factor receptor (p75). During HSC activation, activation of NF-κB increases and with it the HSC acquires a proliferative phenotype. The response of stellate cells to TNF-α depends on relative levels of NF-κB activation. Without active NF-κB, exposure of HSC to TNF-α promotes apoptosis. In the presence of NF-κB activation exposure to TNF-α promotes HSC proliferation (Lang et al. 2000).

1.10.9.3 Inhibition of NF-κB activity may be achieved by exposure to the fungal metabolite gliotoxin. This has been shown to promote apoptosis in rat and human hepatic stellate cells and reduces fibrosis in experimental models of liver fibrosis (Wright et al. 2001). It unfortunately also promotes apoptosis in other cells that require NF-κB for survival. Indeed, the thymus of animals given gliotoxin also undergo apoptosis which would impact dramatically on the immune system. This treatment could potentially prove effective if a liver specific means for drug delivery were available (see below).

1.10.9.4 Other agents that have been shown to promote apoptosis of hepatic stellate cells are Fas ligand via its receptor Fas (Saile et al. 1997), Nerve growth factor via its receptor p75 (Trim et al. 2000), 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (Li et al. 2001) and benzodiazepines via the peripheral benzodiazepine receptor (Fischer et al. 2001). These are potential anti fibrotic agents for further investigation. One problem with this approach is illustrated by gliotoxin, that is because apoptosis is a integral part of all cell populations within the body a systemically administered treatment may cause unwanted apoptosis in other parts of the body, in this case the immune system.

1.10.10 Agents directed to reduce collagen synthesis

1.10.10.1 During chronic liver injury the quantity of fibrillar collagens (types I and III) increase in the liver. This has led to a considerable volume of research into the regulation of collagen synthesis. Collagen type I synthesis involves multiple steps from gene transcription, messenger RNA stabilisation, translation and post translational modification. An interesting feature of mature collagen fibrils is they have a triple helical structure. The stability of this structure is dependent in part on the presence of hydroxyproline residues within the amino acid chain which allows hydrogen bonding to occur between amino acid chains. Hydroxyproline is synthesised within cells from proline by the enzyme Prolyl-4 hydroxylase. Inhibitors of this enzyme include HOE077 and S 4682. Both have been shown to prevent HSC activation and liver fibrosis in experimental animal models of liver fibrosis (Sakaida et al. 1999; Wang et al. 1998; Sakaida et al. 1996; Matsumura et al. 1997; Bickel et al. 1998).

1.10.10.2 Stimulation of the renin-angiotensin system promotes collagen synthesis via aldosterone release and stimulation of the mineralocorticoid receptor. Inhibition of this system has been demonstrated to reduce human cardiac and renal fibrosis (Matsusaka et al. 1999; Klahr and Morrissey 2000). In the bile duct ligation model of liver fibrosis the angiotensin-1 receptor antagonist losartan reduced liver fibrosis (Jonsson et al. 2001), while the angiotensin converting enzyme inhibitor enalapril also reduced liver fibrosis in the thioacetamide model of liver fibrosis (Camino 2001). The long term effect on liver fibrosis by blockade of the renin-angiotensin system has not been studied yet in humans.

1.10.10.3 Endothelin-1 (ET-1) is another vasoconstrictor that has been investigated in activated stellate cells. It acts via two subtypes of cell surface receptor called ET_A and ET_B. Endothelin-1 is over expressed in human liver cirrhosis and has multiple actions on activated stellate cells and appears to be profibrogenic (Pinzani et al. 1996). A selective ET_A antagonist has been shown to prevent fibrosis in the bile duct ligation animal model of liver fibrosis (Cho et al. 2000).

1.10.10.4 Another agent that directly reduces collagen I gene expression is an anti-microbial compound called halofuginone. When given with liver injury development of liver fibrosis is markedly reduced in animal models with this compound (Pines et al. 1997). Interferon gamma also reduces activation and collagen synthesis by stellate cells and reduces fibrosis in experimental animal models (Rockey et al. 1992; Baroni et al. 1996; Mallat et al. 1995). Furthermore, mice that lack Interferon gamma have an increased tendency to hepatic fibrosis after liver injury (Shi et al. 1997). Clinical trials of Interferon gamma are awaited. Interferon alpha has been shown to have an anti fibrotic effect when given to patients with chronic hepatitis C virus infection without clearance of the virus itself indicating that it has an anti fibrotic effect independent of whether the treatment clears the virus (Poynard et al. 1999)

1.10.10.5 TGFβ plays an important role in the pathogenesis of liver fibrosis by increasing collagen synthesis. Briefly, following liver injury inflammatory cells and activated Kupffer cells cause release of latent TGFβ from its binding protein in the local extracellular matrix, to yield bioactive TGFβ. This acts on activated stellate cell TGFβ surface receptors (Friedman and Yamasaki 1991) and through the Smad protein pathway and new gene transcription there is increase in collagen synthesis, reduction in synthesis of matrix degrading MMPs and increase in TIMP-1 expression (Heldin et al. 1997; Gunther et al. 1994).

1.10.10.6 Several inhibitors of the TGFβ pathway have been shown to be effective in experimental models of liver fibrosis. These include the serine protease inhibitor Camostat mesilate which prevents release of latent TGFβ from its binding protein (Okuno et al. 1998), soluble type II TGFβ receptors (George et al. 1998) and a dominant negative type II TGFβ receptor (Qi et al. 1999). One concern over this strategy has been the prediction that blockade of the TGFβ pathway could promote neoplasia. Therapeutic intervention of human liver fibrosis by inhibiting the TGFβ pathway has yet to be investigated.

1.10.10.7 Another interesting feature of collagen I synthesis is the mechanism by which its messenger RNA is stabilised via a novel 5' stem-loop with a binding protein (Stefanovic et al. 2000; Stefanovic et al. 1997). This occurs only in activated hepatic stellate cells and may prove to be a novel target for reducing collagen I protein synthesis.

1.10.11 Agents directed to promote collagen degradation

1.10.11.1 Collagen degradation is largely dependent on local MMP activity. MMPs are activated by other proteins (for example uPA) and some other MMPs. Activity of MMPs are tightly regulated by TIMPs (see later). One difficulty is that extracellular matrix turnover occurs slowly throughout the body. Any modulation of matrix turnover by targeting MMPs, TIMPs, uPA or other regulators would need to be liver specific if it is to avoid systemic adverse effects (for example the development of cataracts or tendon pain). UPA has been used to modify extracellular matrix turnover in an experimental model of liver cirrhosis. UPA is a facilitator of the matrix proteolytic cascade. This protein was expressed via a replication-deficient adenoviral vector coding for a functional form of uPA in the liver. A single exposure of this adenovirus to cirrhotic rats caused increased MMP-2 activity and reversal of liver fibrosis (Salgado et al. 2000).

1.10.11.2 TIMP-1 directly inhibits MMP activity. TIMP-1 was the target in an animal model of liver fibrosis. A TIMP-1 antisense phosphothiolated oligonucleotide was used to reduce TIMP-1 in cirrhotic rats. This did not appear to have an effect on improving liver fibrosis however it is not clear if the antisense to TIMP-1 was effective in vivo (Nie et al. 1999). In contrast, reducing TIMP-1 by a different method with an antisense TIMP-1 expressing plasmid increased collagenase activity and improved liver fibrosis in the pig serum induced model of liver fibrosis (Wang et al. 2002). These studies demonstrate that the method of reducing TIMP-1 in vivo is important if it is to be exploited as a target for therapy.

1.10.11.3 Zinc is known to be a cofactor necessary for MMP activity and collagen degradation. Dietary supplementation of zinc to rats has been shown to reduce Prolyl-4 hydroxylase activity in the alcohol model of experimental liver fibrosis and the carbon tetrachloride model of liver fibrosis (Gimenez et al. 1992; Gimenez et al. 1994).

1.10.12 Methods of targeting activated hepatic stellate cells in vivo

1.10.12.1 As described above, many of the therapeutic agents are likely to have adverse systemic side effects. These might be reduced or even eliminated if the drug were targeted directly to the liver or the HSC itself. Two methods have shown some promise. They made use of the unique expression of the Mannose-6 Phosphate receptor and the type VI collagen receptor present on the activated hepatic stellate cell (Beljaars et al. 1999; Beljaars et al. 2000). Both receptors have been shown to be able to direct experimental drug therapy to the liver. Although yet to be translated into human clinical studies, these reports clearly suggest that some optimism is appropriate for the future of targeted liver therapies.

1.10.13 Quantification of liver fibrosis using serum markers

1.10.13.1 One problem involved in evaluating changes in liver fibrosis is that traditionally this has been quantified by liver biopsy specimens. In the animal models the whole liver can be used for this purpose. In the context of clinical trials in humans, liver biopsy specimens are used. Liver biopsy is not without its own morbidity and mortality rate and sampling bias. Current radiological techniques are too insensitive to monitor the effectiveness of anti fibrotic therapies. Previously, the measurement of individual serum markers of liver fibrosis demonstrated significant limitations in ability to predict and stage liver fibrosis. More recent studies using a panel of several serum markers of liver fibrosis have been evaluated with some promising candidates that could be used to allow routine monitoring of liver fibrosis progression or regression in the future.

1.10.13.2 Serum hyaluronan, type III procollagen peptide and MMP-2 have been shown to reflect the degree of liver fibrosis and reflect response to therapy in patients with hepatitis C virus infection (Guechot et al. 1995; Suou et al. 1995; Kasahara et al. 1997). Serum TIMP-1 level from patients with acute viral hepatitis, cirrhosis, alcoholic hepatitis, and alcoholic cirrhosis has been shown to be significantly elevated compared to controls (Ueno et al. 1996). Furthermore, serum TIMP-1 levels correlated well with histological levels of liver fibrosis and inflammation. However, TIMP-1 does not appear to reflect levels of fibrosis in patients with alcoholic liver cirrhosis (Tsutsumi et al. 1996). While serum markers for hepatitis C virus for monitoring response to therapy have been identified, a single marker that reflects the degree of inflammation and fibrosis in multiple types of chronic liver disease is not yet available.

1.10.14 Factors known to influence hepatocyte regeneration.

1.10.14.1 The focus has so far been mainly on the important role of the HSC in liver fibrosis. It is however important to remember that one of the key questions in hepatology is what determines whether chronic liver injury results in progression of liver disease or resolution and liver regeneration. Liver fibrosis in humans has many causes (Friedman 1993) and there are common themes to be observed. Firstly, there are examples of human liver disease demonstrating evidence of resolution of liver fibrosis provided the liver injury is removed as has been previously discussed (Wanless et al. 2000; Hammel et al. 2001; Dufour et al. 1997; Schiff et al. 2000; Shiratori et al. 2000; Poynard et al. 2000). Secondly, the capacity for the liver to regenerate following injury depends on many factors. The ability of hepatocytes and non parenchymal cells to proliferate during the process of regeneration is of central importance. The concept of hepatocyte proliferative arrest has been studied by many research groups. In human liver cirrhosis, decrease in hepatocyte proliferation and senescence of hepatocyte been described in patients with a range of chronic liver disease (Weimann et al. 2002). Furthermore, the reserve or ability of resident hepatocytes to undergo cellular proliferation has been linked to several aetiological factors including altered hepatocyte matrix interactions (Friedman 1993), the role of stellate cell activation, growth inhibition of hepatocytes by TGFbeta (Milani et al. 1992b), and critical telomere shortening in the hepatocytes themselves (Rudolph et al. 2000).

1.10.14.2 We know from our current clinical practice that for some patients with chronic liver injury the phenomenon of liver regeneration and resolution has been overwhelmed and does not occur to any significant degree. Such patients may deteriorate clinically and may eventually require liver transplantation or suffer the natural history of their liver disease with its complications namely infection, portal hypertension, bleeding and encephalopathy. A greater understanding of the important elements contributing to or preventing liver regeneration will help to highlight potential therapeutic targets of the future.

1.10.14.3 Hepatocyte growth factor is a promising agent that promotes proliferation of hepatocytes and has been shown to improve liver fibrosis in experimental liver fibrosis in rats (Ueki et al. 1999). Again translating these observations in animal experiments into practice in

humans involves resolution of some safety issues most notably its potential risk of carcinogenesis.

1.10.14.4 Telomerase is a good example of one of the many potential therapeutic targets that have been studied in chronic liver disease. Telomerase is the enzyme responsible for maintaining telomere function in eukaryotic cells. Telomeres are nucleoproteins that are found at the ends of eukaryotic chromosomes. With each cellular division, the telomere length reduces and ultimately limits the life span of human cells (Holt and Shay 1999). Once cell telomeres reach a certain minimum size then the cells either eventually become senescent or undergo apoptosis. There are a number of hypotheses to explain why progression to end stage liver disease occurs. The telomere hypothesis of liver cirrhosis suggests that during chronic liver injury wave after wave of hepatocyte destruction and spontaneous regeneration lead to telomere shortening in the remaining hepatocytes in patients with liver cirrhosis. This would eventually lead to a resident hepatocyte population with replicative senescence. Senescent cells are unable to divide thereby limiting the further potential regeneration of the liver following subsequent liver injury.

1.10.14.5 There are a number of elegant animal studies using transgenic mice that have shown data in support of this hypothesis. Firstly, telomerase deficient mice (mTR-/-) develop shortened telomeres with successive generations of mice. These mice demonstrate less liver regeneration following partial hepatectomy compared to wild type mice. Secondly, mice with dysfunctional telomeres are associated with defects in liver regeneration and show an accelerated development of liver cirrhosis in response to chronic liver injury. Thirdly and finally, restoration of telomerase using an adenovirus vector restores telomerase activity in mTR-/- mice with associated restoration in telomere function, reduction in cirrhosis and improved liver function (Rudolph et al. 2000a & b).

1.10.14.6 A number of human studies have presented data in support of the telomere hypothesis of liver cirrhosis. In the study by Weimann et al. 2002 hepatocyte telomere shortening and senescence correlated with progression of fibrosis in cirrhosis samples from a wide array of human liver diseases including autoimmune hepatitis, alcoholic liver disease, primary sclerosing cholangitis and primary biliary cirrhosis. Together, these studies indicate that progression of liver cirrhosis to end stage liver disease at least in part is associated with

telomere shortening and loss of hepatocyte proliferative reserve and may represent the point of no return for patients with liver cirrhosis. With this in mind it is interesting to speculate how this knowledge might be used to help patients with end stage liver disease. It is reasonable to assume that if restoration of telomerase activity could be achieved by a targeted gene therapy then hepatocyte telomere function would be improved and the proliferative reserve of hepatocytes could be restored thus inhibiting liver cirrhosis progression in humans. One problem with this approach is that telomerase overexpression has been demonstrated in cancers (Greenberg et al. 1999). Furthermore, 80% of hepatomas exhibit telomerase activity (Suda et al. 1998).

1.10.14.7 Overall, exploiting telomerase as a potential gene therapy might have benefits in terms of restoring some normal hepatocyte functions but at the risk of carcinogenesis in the long term. There are other unanswered questions in relation to the telomere hypothesis. These include whether non parenchymal cell senescence predisposes to irreversible or progressive fibrotic change. The results from the Weinmann study suggest that the effect of telomere shortening is predominantly in the hepatocytes. Recovery from fibrosis or cirrhosis involves apoptosis of hepatic stellate cells and matrix metalloproteinase mediated matrix degradation. During this process the manner in which signalling between senescent, non senescent hepatocytes and hepatic stellate cells may provide important clues to the regulation of hepatic stellate cell numbers and matrix degradation in liver fibrosis. Gradually our greater understanding of the observed differences in those with progressive liver disease compared to those with stable or resolving liver disease will help direct rational design of future therapies for this difficult problem.

1.10.14.8 Another important factor known to influence hepatocyte regeneration is the presence of type I collagen itself. Recent work has shown that the change in hepatocyte matrix interaction observed in liver fibrosis appears to reduce the ability of the resident hepatocyte regeneration (Issa et al. 2003). In this study by Issa et al. mice bearing a mutated type I collagen that is resistant to degradation by MMPs were used in an experimental model of liver fibrosis and spontaneous resolution. Wild type mice under went spontaneous resolution of fibrosis over 28 days following injury for 8 weeks with carbon tetrachoride injection. In contrast, mice bearing the mutant collagen (r/r mutants) had considerably less evidence of fibrosis resolution. An unanticipated finding of this study was also that the

population of HSC remained elevated in the r/r mutant mice during recovery. Furthermore, there was less hepatocyte regeneration observed in the r/r mice. This data suggests that type I collagen influences hepatocyte regeneration.

1.10.15 The universally effective anti fibrotic drug is still to be developed

The effective and ideal anti fibrotic therapy should include the following characteristics:

1. It should effectively reduce excess collagen within the liver without affecting extracellular matrix turnover elsewhere in the body.
2. It should be liver specific: it will need to have a useful and safe drug carrier.
3. It should have minimal side effects on existing hepatic function, the cardiovascular system and blood coagulation system.
4. It should be tolerated by the immune system rather than influencing its activity.
5. Single therapy working at multiple different mechanistic levels.
6. Ideally a single episode of treatment rather than chronic administration.
7. It should be cost effective and suitable for outpatient treatment.
8. It should improve not just the performance of the liver but also the quality of life of the patient in toto.

At the present time there is no known treatment for chronic liver fibrosis or cirrhosis that meets these criteria. However the knowledge base relating to the pathophysiology of hepatic fibrosis is such that novel effective treatments will likely be developed in the near future.

1.10.16 Conclusions regarding the current anti-fibrotic agents that are in development

1.10.16.1 The detailed understanding of the cell biology of the activated hepatic stellate cell has paved the way to the rational design of novel anti fibrotic therapies. A number of strategies have been shown to be effective in vitro and in animal models. The ideal antifibrotic agent is yet to come, nevertheless such an agent will inevitably be developed given the multiple therapeutic targets that are known within stellate cell biology. In addition it is hoped that the resolution of fibrosis should be quantifiable by serum markers rather than liver biopsy. The results of the clinical trial on interferon-γ and the new TGF-β inhibitors in human liver fibrosis are awaited and will probably be the next anti fibrotics to come from the laboratory bench to the bedside in the immediate future.

1.11 Clinical evidence for reversibility of liver fibrosis.

1.11.1 Traditionally, liver fibrosis and cirrhosis have been considered at best irreversible and at worst, relentlessly progressive. However, throughout the last 30 years there have been isolated reports of small numbers of patients in whom successful treatment of the underlying disease process has resulted in an improvement in liver histology sometimes with a dramatic restitution of normal architecture. These diseases include haemochromatosis responding to vene-section (Powell et al. 1980), eradication of viral infection with Interferon (Poynard et al. 2000), the successful use of immuno-suppression in auto-immune chronic active hepatitis (Dufour et al. 1997) and most recently, successful decompression of the biliary tree in the presence of a secondary biliary fibrosis (Hammel et al. 2001). In many of these examples, the resolution may take many years, but it is associated with the return of normal or near-normal architecture. By definition, this change must be associated with the reduction in the overall numbers of activated myofibroblast-like hepatic stellate cells or a critical change in their activation status.

1.11.2 In none of these studies of human pathology have the dynamics of the non-parenchymal cell components been studied. However, good evidence for resolution of injury being associated with a loss of activated stellate cells is provided by the study of acute paracetamol-induced liver injury (Mathew et al. 1994). In this report, the dynamics of stellate cell numbers were studied both in the acute context and in a follow-up biopsy after spontaneous resolution. Using α-smooth muscle actin as a marker for activated for myofibroblast-like hepatic stellate cells these workers demonstrated that on follow-up biopsy histological resolution was associated with a loss of these actin-expressing cells. Thus, this study provides direct evidence that resolution of injury is associated with a reduction in the number of α-smooth muscle actin positive myofibroblast-like cells. However, no clear underlying mechanism was demonstrated. Whilst these studies have been criticised in the past because they rely on needle biopsy specimens, leading to significant concerns over sampling error, the sheer number of them and the presence now of reports in which larger wedge biopsies or explants of tissues were studied, suggest that this reversal of fibrosis is a real phenomenon.

1.11.3 Convincing evidence for a capacity to remodel fibrosis even in advanced fibrotic liver is provided by the detailed work undertaken by Wanless and colleagues on explanted livers

(Wanless et al. 2000). In this study, clear evidence for matrix degradation and remodelling of fibrotic septa was demonstrated in explanted liver removed at transplantation. As noted above, with the exception of the study of acute paracetamol injury none of these studies directly address the population dynamics or fate of activated myofibroblast-like hepatic stellate cells. Nevertheless, collectively these data support the hypothesis that the capacity for matrix degradation exists even in comparatively advanced liver fibrosis. Moreover that a resolution of injury results in an overall decrease in numbers of activated stellate cells.

1.12 Spontaneous recovery from liver fibrosis by apoptosis of hepatic stellate cells

1.12.1 The development of models of recovery of fibrosis induced by both parenchymal and biliary injury in rodents, has provided an essential tool for understanding the role and regulation of activated hepatic stellate cell apoptosis in the resolution of fibrosis. These models have the advantage that control over the extent of fibrosis and the extent of recovery that can be determined by the investigator providing a full picture of the population dynamics of activated hepatic stellate cells during recovery. After bile-duct ligation, rats rapidly develop fibrosis over a period of fourteen to twenty eight days. An established biliary fibrosis is observed after twenty one days. Following bilio-jejunal anastomosis, however, there is a resolution of the injury over a subsequent six week period with a decrease in the overall level of interstitial collagens and a decrease in the number of activated hepatic stellate cells defined by α-smooth muscle actin staining (Issa et al. 2001).

1.12.2 After treatment with a single injection with carbon tetrachloride (CCl_4), there is a rapid increase in numbers of α-smooth muscle actin positive myofibroblast-like hepatic stellate cells, these numbers rapidly decrease if the injury is not repeated. However, if repeated twice-weekly then over four to six weeks fibrosis becomes established with septa bridging liver vascular structures predominantly central vein to central vein. This injury is however entirely reversible and over a period of 28 days after the last dose of CCl_4. There is a resolution of the fibrotic change with a decrease in expression of collagen type 1, TIMP-1 and a concurrent increase in collagenolytic activity associated with which matrix degradation is demonstrable both biochemically and in terms of histological resolution (Figure 1.3) (Iredale et al. 1998).

1.12.3 Analysis of whole liver hydroxyproline (a surrogate marker of collagen) demonstrates that during spontaneous recovery the amount of collagen returns to a level comparable to normal untreated liver. Quantification of apoptosis by the TUNEL technique showed that there was a significant increase in apoptosis of HSC during the recovery phase suggesting that apoptosis of activated HSC may vitally contribute to the resolution of fibrosis. Quantification of whole liver RNA demonstrated a rapid decrease in expression of TIMP-1 and TIMP-2 while the expression of collagenase mRNA remained at levels comparable to peak fibrosis. Collagenase activity in liver homogenates increased through recovery (Iredale et al. 1998). Together this pattern of dynamic changes with decreasing TIMP-1, increasing MMP activity, loss of HSC by apoptosis and loss of type I collagen suggest that the balance of MMP activity versus TIMP-1 activity may mediate HSC survival.

FIGURE 1.3 SPONTANEOUS RESOLUTION OF EXPERIMENTAL LIVER FIBROSIS.

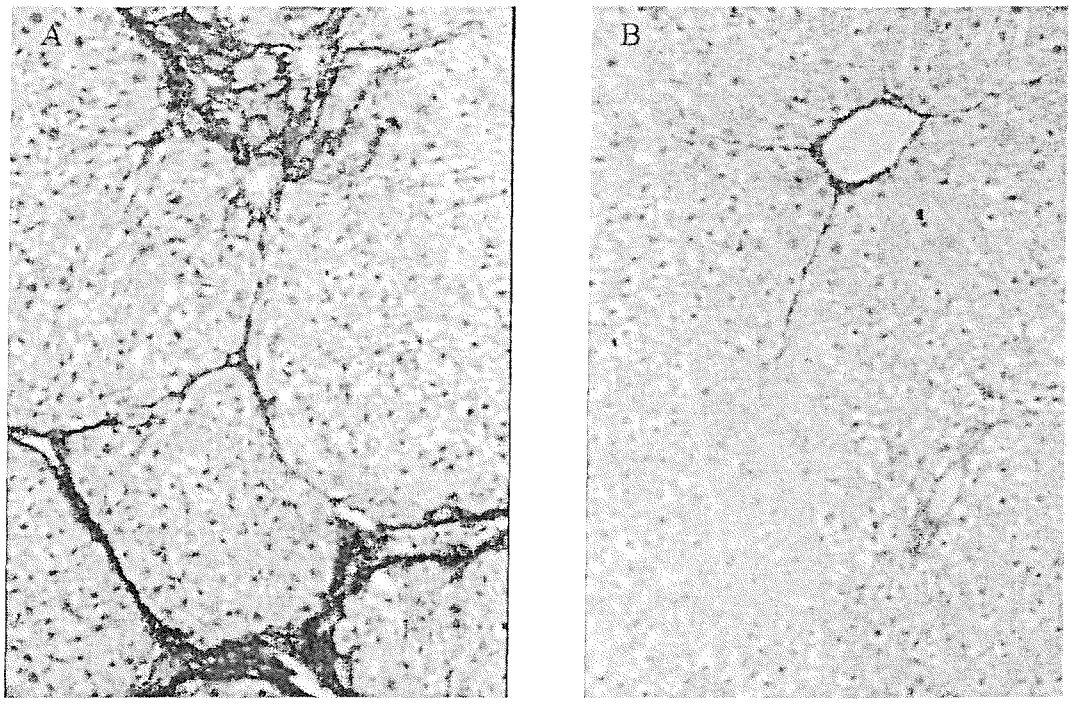


Figure 1.3. (A) Histological sections of rat liver treated twice weekly with carbon tetrachloride for 4 weeks demonstrated an advanced septal fibrosis at time 0 (peak fibrosis). (B) After 28 days of spontaneous recovery liver histology returns to near normal. Reproduced from Iredale et al. 1998.

1.12.4 Both the bile-duct ligation-bilio-jejunal anastomosis and CCl_4 reversal model demonstrate a progressive decrease in the number of myofibroblast-like cells determined by α-smooth muscle actin staining during the recovery period. Whilst there is a dramatic decrease in these number of cells even after histological resolution the numbers seen are still in excess of those observed in untreated livers. Histological analysis of liver sections from these models during the first week of recovery, when loss of α-smooth muscle actin positive hepatic stellate cells is maximal, demonstrate that there is evidence of hepatic stellate cell apoptosis on the basis of morphology using standard Haematoxylin & Eosin stains, morphology after staining with propridium iodide and after specific identification of fragmented DNA in apoptotic cells by TUNEL staining (Figure 1.4) (Iredale et al. 1998; Issa and Williams 2001). Together, the data from these models suggest that apoptosis of myofibroblast-like activated hepatic stellate cell is a vital component of recovery from fibrosis. This process serves the dual function of removing the major matrix producing cell whilst, at the same time, removing the cells that are expressing TIMPs and thus inhibiting matrix degradation.

FIGURE 1.4. EXAMPLES OF APOPTOTIC HSC.

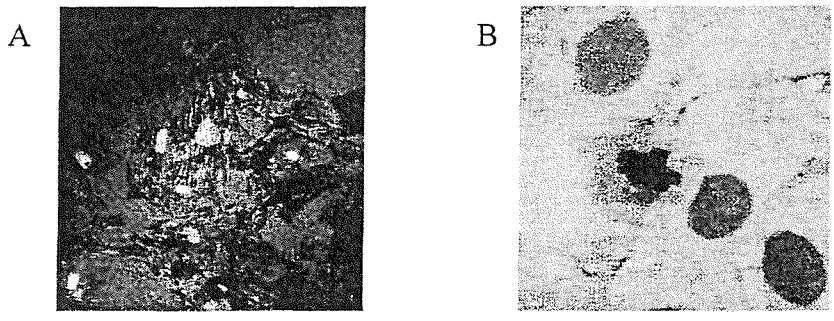


Figure 1.4. (A) Fixed HSC stained with propidium iodide and photographed by confocal microscopy. The condensed nuclei of apoptotic HSC show as intensely stained nuclei and contrast with the larger nuclei of non apoptotic nuclei. (B) HSC giemsa staining show a range of morphological features that characterise apoptosis with nuclear condensation, cytoplasmic blebbing and nuclear fragmentation. Taken from Iredale et al. 1998.

1.12.5 Direct evidence for the beneficial effect of removing activated hepatic stellate cells during recovery from liver injury has recently been demonstrated. The fungal metabolite gliotoxin induces apoptosis of activated myofibroblast-like hepatic stellate cells in culture

(Wright et al. 2001). Within two to three hours of incubation with gliotoxin, culture activated HSC demonstrate morphological alterations typical of apoptosis, increase in caspase-3 activity and oligo-nucleosomal fragmentation of DNA. When gliotoxin is administered to rats together with the final dose of CCl_4 after six week's of CCl_4 twice weekly, a dramatic increase of apoptotic HSC was observed. This resulted in a significant decrease in the overall numbers of activated hepatic stellate cells within 24 hours. In addition, there was a significant reduction in the width of fibrotic septa measured at the mid-centrilobular points. Taken together these data provide powerful evidence that removal of activated hepatic stellate cells through apoptosis facilitates the process of histological restitution by the mechanism outlined above. One interesting observation that this data highlights is that the apoptotic hepatic stellate cells may not be the major source of the MMPs ultimately degrading the fibrotic matrix.

1.12.6 Models in other cell-culture systems and human diseases suggest that this loss of myofibroblast is a common phenomenon in the resolution of fibrotic injury. Parallels exist in experimental glomerulonephritis (Mizuno-Horikawa Y 2001), and the resolution of wound-healing in the skin (Clark 1993). Indeed, the role of the hepatic stellate cell in liver wound healing may be considered a paradigm for wound healing in its most general form.

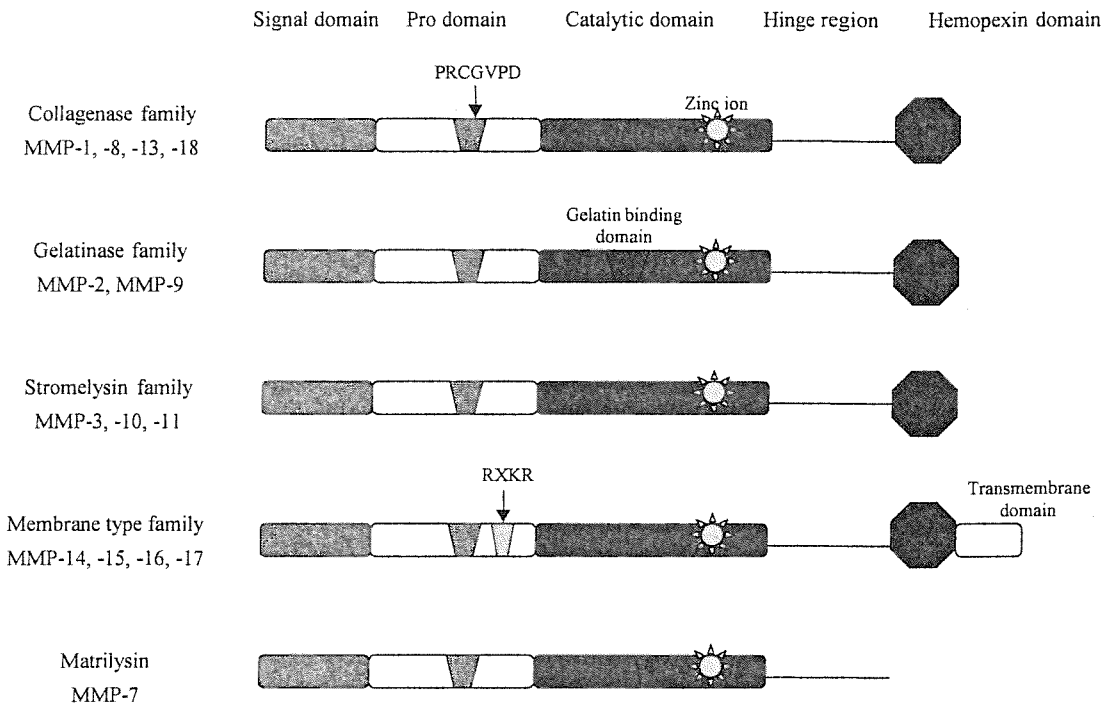
1.13 The Matrix Metalloproteinases

1.13.1 MMP structure and function

The matrix metalloproteinases (MMPs) are the most important enzymes involved in matrix degradation in the extracellular space. They are zinc and calcium dependent and there are at least 28 members of this family (Sternlicht and Werb 2001). They are named either by their common names or by sequential nomenclature. They have a modular domain structure (Figure 1.5). Common to all MMPs, is an N-terminal signal sequence ("pre" domain) that is removed after it directs synthesis to the endoplasmic reticulum. Most MMPs are secreted but six contain transmembrane domains and are expressed as cell surface enzymes ("Membrane type" MMPs or MT-MMPs). The "pre" domain is followed by the "pro" domain which protects the catalytic site and maintains enzyme latency until it is removed. The pro domain contains a conserved cysteine that chelates the active zinc site which is located in the catalytic domain. Once the pro peptide is removed the catalytic domain becomes active. The generally

conserved site PRCGVP around the zinc chelating site has been called the "cysteine switch." A subset of MMPs (including the membrane type MMPs) contain a basic prohormone convertase cleavage sequence (RXKR). This is thought to be cleaved by the Furin family of enzymes. All MMPs have a hemopexin domain (except MMP-7, 23 and 26). This is connected to the catalytic domain by a hinge region. The hemopexin domain influences TIMP and substrate binding (Overall 2001) and membrane activation of the MMP. Gelatinases A and B (MMP-2 and MMP-9 respectively) also have a collagen binding domain.

FIGURE 1.5 DOMAIN STRUCTURE OF MATRIX METALLOPROTEINASES



1.13.2 Regulation of MMP activity

For MMPs to carry out their functions correctly, MMPs must be present in the right cell type and pericellular location, at the right time, in the right amount, and they must be activated or inhibited appropriately. For this to occur, MMPs are tightly regulated at the transcriptional and post-translational levels, also the MMPs are controlled at the protein level via their activators, inhibitors and cell surface location.

1.13.2.1 *Transcriptional Regulation of MMPs* - The biological function of individual MMPs is determined by their differential patterns of expression. Most MMPs are closely regulated at the level of transcription with the notable exception of MMP-2. MMP-2 is often constitutively

expressed and controlled through a unique mechanism of enzyme activation (Strongin et al. 1995). Indeed, data indicate that basal expression of MMP-2, MMP-14 (MT1-MMP) and TIMP-2 is co-regulated (Lohi et al. 2000). This is consistent with their co-operation during MMP-2 activation at the cell surface (see later). Other MMPs are regulated by many stimulatory and suppressive factors. For example, type I collagen acts as a ligand for the discoidin domain containing receptor-like tyrosine kinase that can induce MMP-1 expression (Vogel et al. 1997, Shrivastava et al. 1997). Thus MMP-1 expression can be induced by its own substrate. MMP expression is regulated by many cytokines and growth factors for example TGF-β, NGF, PDGF and interferon. These stimuli operate via the *c-fos* and *c-jun* proto-oncogene products and bind to the activator protein-1 (AP-1) sites within several MMP gene promoters. Other more novel transcription factors have been shown to be involved in some specific MMP promoters. For example, a β-catenin regulated LEF/TCF recognition site near the MMP-7 transcription start site has been described (Crawford et al. 1999). Also, a functioning p53-binding site has been found in the MMP-2 gene promoter (Bian and Sun 1997) suggesting that MMP-2 may have a role in apoptosis.

1.13.2.2 *Post transcriptional regulation* - MMP expression is also influenced by post-transcriptional mechanisms. For example mRNAs for MMP-1 and MMP-13 are stabilized by phorbol esters and EGF, also mRNA for MMP-13 are stabilized by PDGF and glucocorticoids and destabilized by TGF-β (Delany et al. 1995; Vincenti 2001).

1.13.2.3 *Regulation of MMP secretion* - Most MMPs are constitutively secreted once they have been translated. In some circumstances there is evidence that some cell types demonstrate some secretory control. For example, MMP-8 (neutrophil collagenase) and MMP-9 are made in maturing granulocytes in the bone marrow and are stored in granules of the circulating neutrophils. Following neutophil activation by inflammatory mediators, there is degranulation and release of the stored MMPs (Hasty et al. 1990).

1.13.2.4 *Activation of latent MMPs* - Like most proteolytic enzymes, MMPs are first synthesised as a proenzyme or zymogen. The MMP latency is maintained by the "cysteine switch" mechanism. This is made up of an unpaired cysteine sulfhydryl group near the C-terminal end of the propeptide domain. This sulfhydral group forms the ligand for the active zinc ion present in the catalytic domain. Activation requires the cysteine switch to be opened

by proteolytic removal of the propeptide domain (Van Wart and Birkedal-Hansen 1990). Once the propeptide has been removed the sulfhydral group is replaced with a water molecule that is then utilised to attack peptide bonds of MMP substrates.

Another mode of activation is demonstrated by the MMPs that contain the RXKR furin-like enzyme motif. MMP-11, MMP-27 and the MT-MMPs contain this motif and are activated within the cell by serine proteases before they reach the cell membrane (Pei and Weiss 1995). At the cell surface, active MT1-MMP is inhibited by TIMP-2 while other TIMPs, most specifically TIMP-1, is less able to inhibit active MT1 MMP. Extracellular activation of most MMPs can occur by another active MMP or by serine proteases for example plasmin (Sternlicht and Werb 2001). MMP-2 is unusual because it is resistant to serine protease activation instead it has a complex activation pathway involving MT1-MMP and TIMP-2 (Strongin et al. 1995). Firstly, a cell surface MT1-MMP binds and is inhibited by TIMP-2. This MT1-MMP/TIMP-2 complex serves as a receptor for proMMP-2. The C-terminal end of TIMP-2 binding to the haemopexin domain of the proMMP-2. Next, an adjacent MT1-MMP undertakes the initial cleavage of proMMP-2 to yield active MMP-2 that is still bound to the MT1-MMP/TIMP-2 complex. Finally, another active MMP-2 molecule cleaves the remaining part of the MMP-2 propeptide to yield fully active MMP-2 (Deryugina et al. 2001)(Figure 1.6). Once activated the active MMP-2 molecule has a number of fates including substrate degrading activity or the formation of a number of MMP-2/TIMP soluble complexes with TIMP-1 or TIMP-2.

FIGURE 1.6. CELL SURFACE ACTIVATION OF MMP-2 BY MT1 MMP (STRONGIN ET AL 1995)

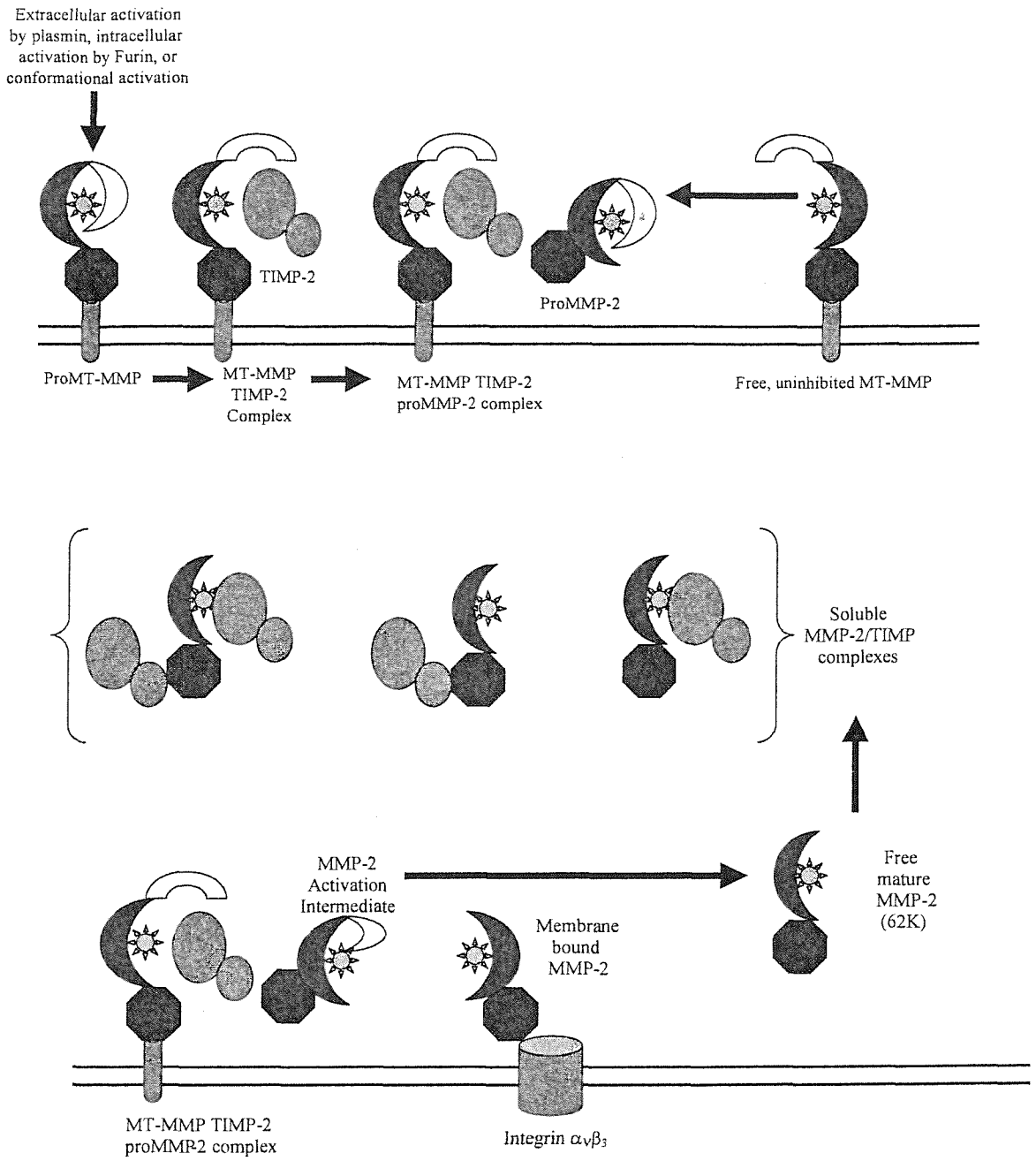


Figure 1.6. The complex activation pathway of MMP-2 involving MT1-MMP and TIMP-2. A cell surface MT1-MMP binds and is inhibited by TIMP-2. This MT1-MMP/TIMP-2 complex serves as a receptor for proMMP-2. The C-terminal end of TIMP-2 binding to the haemopexin domain of the proMMP-2. Next, an adjacent MT1-MMP undertakes the initial cleavage of proMMP-2 to yield active MMP-2 that is still bound to the MT1-MMP/TIMP-2 complex. Finally, another active MMP-2 molecule cleaves the remaining part of the MMP-2 propeptide to yield fully active MMP-2

1.13.2.5 *MMP clearance*-It is self evident that a protease may be able to inactivate itself by autocatalytic digestion. MMPs are known to behave in this way to some extent. Some MMP cleavages inactivate the enzyme while others, for example cleavage of the hemopexin domain generate truncated enzymes that lose their ability to cleave some substrates but retain their ability to cleave others. Autocatalytic processing of MMP-2 C-terminal has been shown to diminish its ability to be inhibited by TIMPs (Itoh et al. 1998). Loss of the haemopexin domain also removes the ability of the MMP to localise to the cell surface. Thus MMP degradation is yet another way that local MMP activity is mediated.

α-2 macroglobulin and thrombospondin 2 (TS2) are also involved in the clearance of MMPs. The α-2 macroglobulin bait region is cleaved by active MMPs. This causes a conformation change that traps the active MMP irreversibly (Sottrup-Jensen and Birkedal-Hansen 1989). The MMP/α-2 macroglobulin complex is eventually endocytosed and permanently cleared. Thrombospondin 2 binds to both latent and active MMP-2. This complex is endocytosed via the low-density lipoprotein receptor related protein (Yang et al. 2001). Mice that are TS2-deficient show a number of connective tissue abnormalities, also fibroblasts from these mice have an adhesion defect that is due to increased MMP-2 levels(Yang et al. 2000). Thus MMPs are tightly regulated by several mechanisms during every part of their life-span from induction to their clearance.

1.13.3 *MMP substrates*-There are numerous MMP substrates that have been identified in vitro. Many, not surprisingly, are extracellular matrix proteins, however there are also non matrix substrates. These include cytokine binding proteins (for example, Insulin-like growth factor binding proteins), other proteases (for example, plasminogen and other MMPs), cytokines (for example, Interleukin 1β), and some cell surface molecules (for example, E-cadherin). The range of potential MMP substrates is in fact legion. Indeed, two screening methods have emerged as a means of identifying potential substrates. For example, monocyte chemoattractant protein-3 (MCP-3) was identified as a substrate for MMP-2 after using the MMP-2 haemopexin domain bait to search for potential binding proteins in yeast two-hybrid screens (McQuibban et al. 2000). Another means of searching for new substrates uses a phage-displayed hexapeptide library to screen for preferred substrate amino acid motifs. This has been under taken for human MMP-13 (Deng et al. 2000) and MMP-2 (Chen et al. 2002). Once the amino acid cleavage motifs are known, protein database searches yield potential new MMP substrates. These candidate proteins can then be tested in vitro with the MMP of

interest. A problem that exists within the literature is that although numerous MMP substrates have been described in vitro, few have been definitively described in vivo.

TABLE 1.1 MMPS AND THEIR SUBSTRATES. Adapted from Benyon 2001

NOMENCLATURE		SUBSTRATES
Interstitial collagenases		
MMP-1	Interstitial collagenase	Collagen III>I, II, VII, VIII, X
MMP-8	Neutrophil collagenase	Collagen I> III, II
MMP-13	Collagenase-3	Collagen II> III, I, VII, X
Gelatinases		
MMP-2	Gelatinase-A (72 kDa type IV collagenase)	Collagen V> IV, I, VII, X, gelatins, elastin, laminin
MMP-9	Gelatinase-B (92 kDa type IV collagenase)	As MMP-2
Stromelysins		
MMP-3	Stromelysin-1	Collagen III, IV, V, IX, gelatins, fibronectin, proteoglycans, laminin, activates MMP-1
MMP-7	Matrilysin	Entactin, gelatin, elastin, fibronectin, vitronectin, laminin, fibrogen
MMP-10	Stromelysin-2	As MMP-3, gelatins III, IV, V> collagens, elastin, aggrecan
MMP-11	Stromelysin-3	Weak activity against matrix proteins
Membrane-type		
MMP-14	MT1-MMP	Activates MMP-2 and MMP-13, collagen I, II, III, fibronectin, vitronectin, gelatin, fibrinogen
MMP-15	MT2-MMP	Fibronectin, tenascin, laminin, aggrecan, perlecan, activates MMP-2
Also MMP-16, MMP-17, MMP-24, MMP25		
Metalloelastase		
MMP-12	Metalloelastase	Elastins, gelatins, collagen IV, laminin, fibronectin, proteoglycan

1.13.4 *Pericellular localisation of MMP activity* - The cell surface of many cells is the location of many events that mediate cell behaviour. Many of these events are created or cancelled via pericellular proteolysis (Werb 1997). Specific means exist that facilitate the co-localisation of MMPs with their substrate. For example, membrane type MMPs present at the cell surface, and collagen binding domains present on MMP-2 and MMP-9 facilitate MMP binding to collagens. Localisation is further facilitated by the presence of cell surface receptors for MMP activating enzymes for example uPA, plasminogen, thrombin and elastase. These systems enhance MMP activation, reduce the influence of MMP inhibitors and concentrate MMPs in the vicinity of their targets enabling discrete pericellular proteolysis (Sternlicht and Werb 2001).

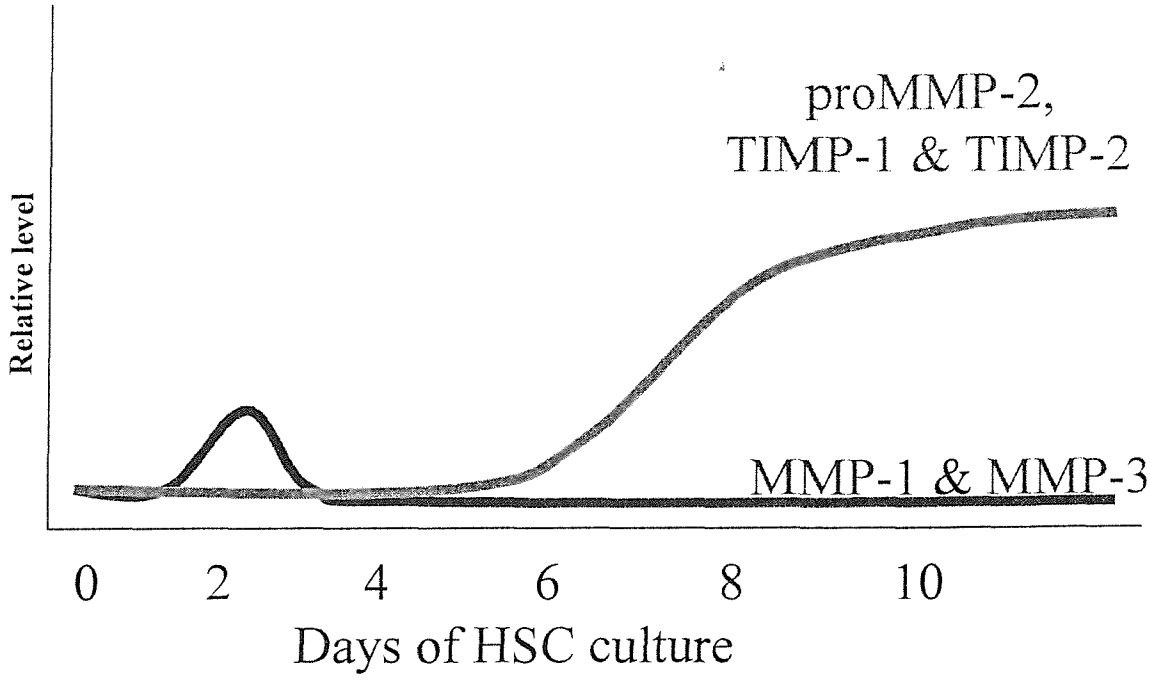
MMP-2 has been shown to bind to integrin αVβ3 and participate in the activation of proMMP-2 at the cell surface (Figure 1.6). This provides another mechanism to localise MMP activity to the cell surface (Deryugina et al. 2001). Similarly, MMP-1 has been demonstrated to bind to the α2 integrin subunit of integrin α2β1 via its haemopexin domain, in contrast MMPs 3 and 13 do not (Stricker et al. 2001). Activated MMP-9 binds to the cell surface hyaluronan receptor (CD44) (Bourguignon et al. 1998) and may mediate the activation of latent TGF-β by MMP-9 (Yu and Stamenkovic 2000).

1.14 Matrix metalloproteinases of the liver

1.14.1 At least six MMPs are known to be active within the liver. These include the collagenases (MMP-1 and MMP-13) (Iredale et al. 1995; Iredale et al. 1996), the gelatinases A and B (MMP-2 and MMP-9) (Arthur et al. 1992; Winwood et al. 1995), stromelysin (MMP-3) (Vyas et al. 1995a)and the membrane type MMPs (MT1-MMP or MMP-14) (Hovell et al. 1995). Each degrade a broad range of substrates including proteoglycans, laminin, gelatins and fibronectin (Table 1.1)(Arthur et al. 1998). In studies of cultured cells, HSC and Kupffer cells have a prominent role in matrix degradation. Expression of MMP from HSCs is variable according to the state of activation. For example early in primary HSC culture there is transient expression of interstitial collagenase (MMP-1). This is spontaneously down regulated after 3-5 days and is undetectable in fully activated HSC (day 7-21)(Vyas et al. 1995a; Herbst et al. 1991). This contrasts with the expression of MMP-2 which is undetectable early in culture but is highly expressed in activated HSCs (Figure 1.4). Kupffer cells synthesise MMP-9, small amounts of MMP-2, and therefore contribute to matrix

degradation (Milani et al. 1994). In contrast, there is no conclusive evidence that hepatocytes release MMPs into the extracellular space (Arthur et al. 1998).

FIGURE 1.7. COMPLEX TEMPORAL PATTERN OF EXPRESSION OF TIMPS AND MMPS WITH HSC ACTIVATION IN TISSUE CULTURE.



1.14.2 ***MMP expression in diseased liver*** - MMP expression has been studied in normal and diseased liver. These have shown different expression of MMPs. For example, MMP-2 (Gelatinase A) expression is low in normal liver but greatly increased in liver disease or experimental induced liver injury (Benyon et al. 1997). This contrasts with interstitial collagenase expression which remains relatively constant in normal, injured or diseased liver (Denhardt et al. 1993). On face value it seems counterintuitive that fibrogenesis occurs in the presence of matrix degrading metalloproteinases. However, current evidence suggests that expressed MMPs may themselves be inhibited by other HSC products. In addition, disruption of cell matrix interactions by MMPs may directly influence HSC activation and proliferation.

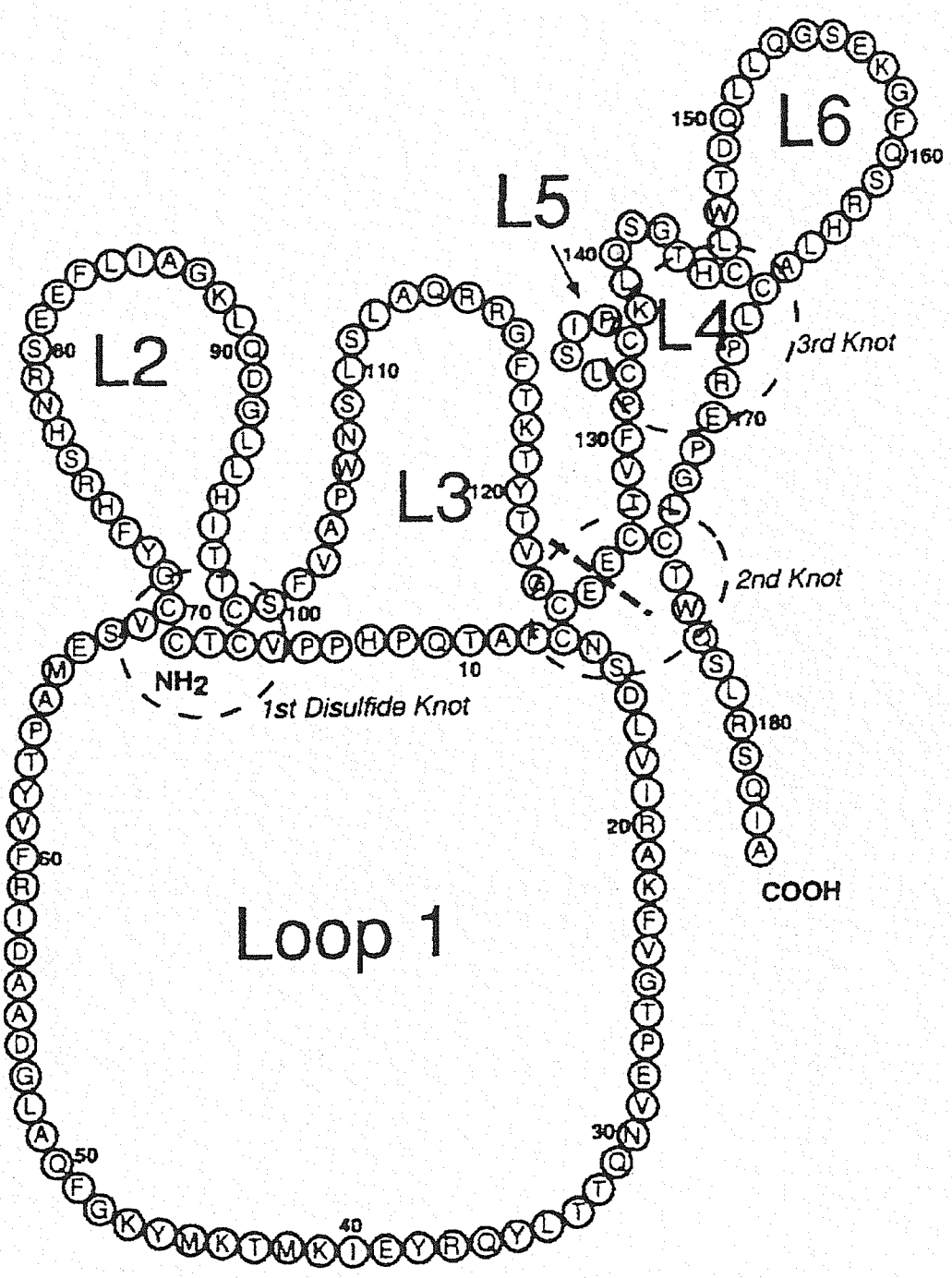
1.14.3 It has recently been demonstrated that MMP-2 (Gelatinase A) may act as an autocrine survival factor for HSCs promoting their proliferation which in turn may promote fibrogenesis (Benyon et al.1999). The mechanisms underlying this effect is unknown but it may be mediated by disruption of normal cell-matrix interactions or via local release of matrix bound profibrotic growth factors. Studies of matrix degrading activities in liver

homogenates have provided insight into the net activity of MMPs in normal and fibrotic livers. Generally, these studies show an increase in collagenase activity early in liver injury while later, as fibrosis progresses, activity declines. Evidence from several models suggests that this change in activity may be due to concurrent expression of metalloproteinase inhibitors, the tissue inhibitors of metalloproteinase (or TIMPs) by HSCs.

1.15 The Tissue inhibitors of metalloproteinase (TIMPs)

1.15.1 Matrix metalloproteinases are inhibited by a family of endogenous inhibitors termed TIMPs. There are now four distinct TIMP members described. Much information already exists about TIMPs 1 and 2 while TIMPs 3 and 4 are less well characterised. As a family, these proteins show common structural motifs with 40% amino acid sequence homology between TIMPs 1 and 2. Both have a looped structure stabilized by six disulfide bonds, the result of 12 conserved cysteine residues (Figure 1.8). The binding of TIMPs to MMPs is essentially irreversible under physiological conditions. This binding is stoichiometric, non-covalent and the TIMP/MMP complexes can be separated. TIMP-1 and TIMP-2 inhibit the active forms of every metalloproteinase (Hayakawa et al. 1992). However, TIMP-2 and TIMP-3 can inhibit MT1-MMP, while TIMP-1 does not. Studies using site directed mutagenesis and recombinant TIMPs indicate that there are two separate domains to the molecule. The N-terminal is necessary for inhibitory activity against active MMPs (Huang et al. 1997). The C-terminal domain is key to the interaction with prometalloproteinases (Murphy et al. 1999). TIMPs demonstrate growth factor activity on many human cell types (described below) and may also have effects on HSCs.

FIGURE 1.8. PRIMARY STRUCTURE AND DISULFIDE BONDS OF HUMAN TIMP-1.



(Reproduced from Bodden et al.1994)

1.15.2 *The evidence that TIMPs have roles in regulating cell survival* - Whilst previous work has emphasised the potential importance of TIMPs to fibrosis via inhibition of matrix degradation, individual TIMPs may regulate cell division and apoptosis independently of this activity. TIMP-1 has been shown in vitro to suppress apoptosis of Burkitt's lymphoma cell lines (Guedez et al. 1998) and human breast epithelial cells (Li et al. 1999). In these studies the anti apoptotic effect of TIMP-1 was reported to be independent of its ability to inhibit MMP activity. Moreover, several neoplasms demonstrate a positive correlation between TIMP-1 and TIMP-2 expression and metastatic spread. This observation is counter intuitive to the previously accepted hypothesis that TIMP-1 mediated inhibition of matrix degradation would retard tumour dissemination (Ree et al. 1997). TIMP-1 was originally identified as a growth factor for myeloid elements and has also been demonstrated to promote fibroblast proliferation (Docherty et al. 1985, Kikuchi et al. 1997). These data suggest that TIMPs may be important regulators of cell growth and apoptosis.

1.15.3 The TIMPs appear to have divergent effects on proliferation and apoptosis in different cell types. For example TIMP-2 acts as a growth factor for mesenchymal cells in rat kidney development (Barasch et al. 1999) while it is a pro apoptotic stimulus for human T lymphocytes (Lim et al. 1999). Adenoviral over expression of TIMP-1, -2 and -3 has been studied in rat aorta smooth muscle cells. Within the same cell type the TIMPs had divergent effects, TIMP-1 over expression had no effect on cell proliferation while TIMP-2 produced a dose dependent reduction in proliferation. This effect was not mimicked by a synthetic matrix metalloproteinase inhibitor. TIMP-3 over expression induced DNA synthesis and promoted apoptosis in myofibroblasts (Baker et al. 1998). In contrast, TIMP-2 over-expression in B16F10 melanoma cells protects these cells from apoptosis but had no effect on proliferation (Valente et al. 1998). The close correlation between the reduction of TIMP-1 and TIMP-2 expression and apoptosis of HSC observed in vivo during recovery from liver fibrosis highlights a possible role for TIMP-1 and TIMP-2 in regulating HSC survival.

1.15.4 Amino acid position number 2 of TIMP-1 is the major determinant of affinity and specificity for MMPs - Recently, a mutated N-TIMP-1 (Threonine number 2 to Glycine or T2G mutant) has been developed which has virtually no MMP inhibitory activity compared to the wild type TIMP-1 (Meng et al. 1999). This mutated TIMP-1 now allows the dissection of any anti or pro apoptotic effect on stellate or other cells to be examined. In particular, this

reagent can be used to determine whether any effects TIMP has on apoptosis or proliferation are due to MMP inhibition or via alternative mechanisms.

1.16 Regulation of stellate cell apoptosis

1.16.1 Apoptosis in cells can be regulated through a series of mechanisms. These include the stimulation of specific families of cell-surface death receptors by particular ligands the best known example of this is the Fas, Fas-Ligand system (Figure 1.9). Binding of a ligand to such cell receptors result in activation of a so-called intracellular death domain with resulting activation of the caspase cascade and ensuing cellular apoptosis. A second pathway relates to the cell mitochondria. Pro and antiapoptotic proteins of the Bcl-2 family are located in the mitochondria membranes (Figure 1.9). Predominance of pro-apoptotic proteins (e.g. bax, bid) results in the formation of homodimers which facilitate the passage of cytochrome C from the mitochondria into the cytoplasm where a complex is formed with APAF-1 (Apoptotic protease activating factor–1). This so-called "apoptosome" complex activates the caspase cascade with resulting apoptosis. The presence of cytochrome C in the cell cytosol is thought to be the point of no return for a cell, such cells rapidly undergo apoptosis. Alternatively, the predominance of anti-apoptotic family members (e.g. Bcl-2) tends to prevent cytochrome release and promotes survival of the cell (Blatt & Glick 2001). Many other proteins have been described to regulate these two basic apoptotic pathways. One example is the inhibitor of apoptosis proteins (IAPs). Current evidence suggests that for both systems the balance of pro and apoptotic factors in any cell determine its fate and susceptibility to apoptotic stimuli or withdrawal of survival stimuli.

1.16.2 A current model, which fits well with observations of hepatic stellate cells, is that myofibroblast-like cells of wound healing are prone to apoptosis as a default pathway. This default position is forestalled provided the cell obtains survival signals. These signals may be derived from the cellular micro-environment in the form of soluble growth factors, cytokines, cell-matrix or cell-cell interactions. Apoptosis ensues when, with resolution of injury, one or more survival stimuli are withdrawn.

FIGURE 1.9 THE BASIC APOPTOSIS PATHWAYS: EXTRINSIC AND INTRINSIC VARIANTS

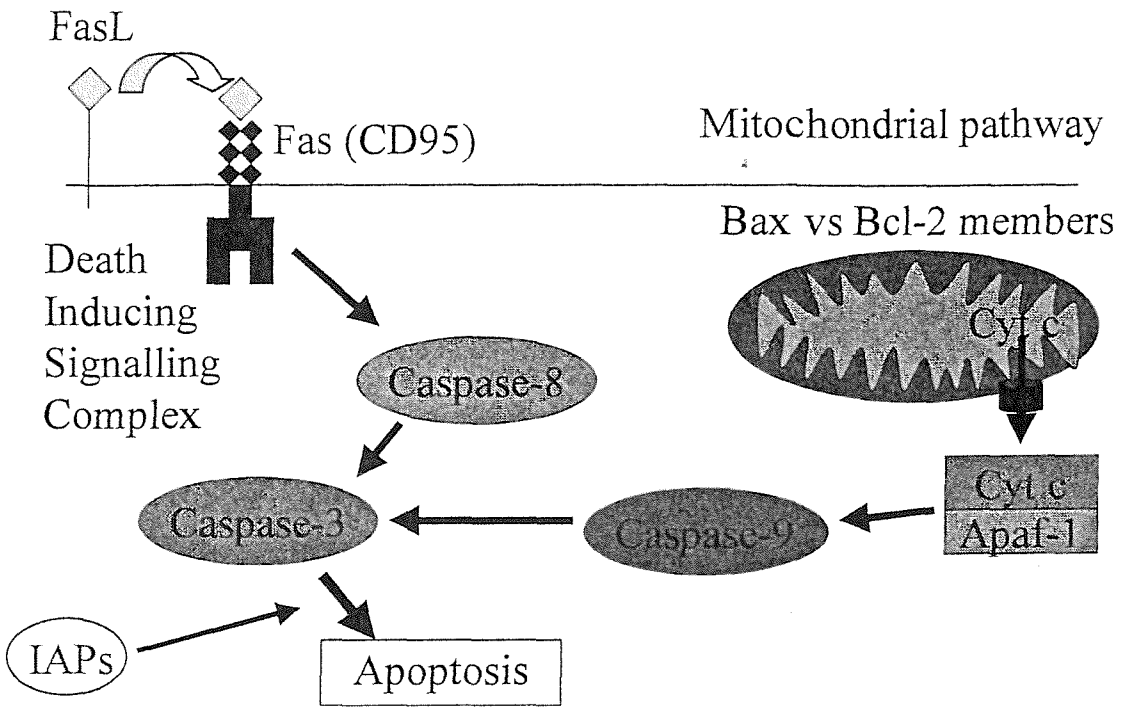


Figure 1.9. The extrinsic apoptotic pathway involves any of a wide variety of cell surface death receptors (for example Fas). Ligation of this receptor with its appropriate ligand (for example Fas ligand) results in assembly of the death inducing signalling complex. This activates the caspase protease cascade with subsequent apoptosis. The intrinsic pathway operates at the level of the mitochondria. A large family Bcl-2-like regulatory proteins maintain a balance on the outer mitochondria membrane. Certain stimuli (for example toxins, UV radiation, and gliotoxin) cause a disturbance in the balance of pro versus anti apoptotic factors. This leads to the formation of a transmembrane pore through which cytochrome C leaks. Following this event there is rapid activation of the caspase cascade and apoptosis ensues.

1.17 Hypothesis and aims

1.17.1 Hypothesis

TIMP-1 and TIMP-2 regulate hepatic stellate survival through inhibition of MMP mediated degradation of protein substrates that provide survival stimuli in the form of cell to cell and cell to matrix interactions.

1.17.2 Aims

1. To determine the effect of TIMP-1 and TIMP-2 on HSC proliferation and apoptosis
2. To determine whether this effect (if any) is through effects on MMP inhibition
3. To determine which MMP or MMPs are involved in HSC apoptosis
4. To determine if a candidate MMP directly effects HSC apoptosis
5. To determine MMP substrates that may mediate HSC survival or apoptosis

Chapter 2

2.1 Materials

All laboratory materials were supplied by Sigma Chemicals (Poole, UK) unless otherwise stated. Molecular biology materials were supplied by Promega (Southampton, UK) unless otherwise stated. A list of the different buffers used in the work is provided in Appendix I.

2.2 Tissue culture methods

2.2.1 Isolation of human and rat hepatic stellate cells.

Appropriate home office licences were obtained and local ethics committee approval obtained before any animal work was undertaken. Rat HSC were extracted from normal rat liver by pronase and collagenase digestion and purified by centrifugal elutriation as described (Arthur et al. 1989). Briefly, after the rat was anaesthetised with an intraperitoneal injection of Sagatal (12mg/100g body weight), the portal vein was cannulated and secured with a suture. The liver was perfused with approximately 200ml of HBSS without calcium at a rate of 15ml/minute and then the liver removed from the carcass. Next enzymatic digestion of the liver was undertaken first with a 100ml of pronase solution (100mg/100ml HBSS with calcium, Roche Diagnostics). This was followed by perfusion with a collagenase solution for approximately 20 minutes (20mg/200ml HBSS with calcium, Roche Diagnostics). Next the liver was transferred to the tissue culture hood, the capsule of the liver cut open, and the liver digest was filtered through a nybolt filter. The crude extract was then washed twice in HBSS with calcium and DNase solution (Roche Diagnostics) and then the cell suspension was made up to 44.4ml and were separated by adding to a density gradient made from 15.6ml HBSS with calcium and 14ml of Optiprep – Density 1.320 g/l (Axis-Shield, Oslo, Norway). This mixture was divided between two sterile 50ml falcon tubes and carefully balanced. The cells were spun in a centrifuge at 1400g for 17 minutes with the brake off. Once the spin had stopped, the stellate cells appeared as a cloud visible immediately beneath the cushion of HBSS with calcium. This layer was carefully removed and washed again in HBSS with calcium and DNase solution. This cell extract was further purified using the centrifugal elutriator. This step was under taken by a laboratory technician who at the time was Mrs Anne Wooler. Typical rat preparation from one liver would yield around 100 million stellate cells with 95% viability and

purity by trypan blue staining and counting in the hemocytometer. Four large squares on the graticule were counted and a mean taken (let this be Y). The number of cells per ml x 10^4 = Y x 1.33 (dilution factor for 25µl of trypan blue in 75µ l of cell suspension).

2.2.2 Isolation of human hepatic stellate cells.

Human HSC were extracted from the margins of normal human liver resected for colonic metastatic disease as previously described (Iredale et al. 1995). Briefly, the liver specimen was collected from the operating theatres and taken to the histopathologist who cut a piece of normal liver from the liver resection specimen. Local ethics committee approval was obtained and prior consent was taken from the patient the day before surgery. A letter was also sent to the patients General Practitioner. The normal liver specimen was taken to the tissue culture hood and cut into fine pieces with scalpels and decanted into a sterile glass bottle. A mixture of collagenase (20mg/20ml HBSS with calcium), pronase (100mg/20ml HBSS with calcium) and 5ml of DNase solution (10mg/20ml HBSS with calcium) were added to the liver. This was then incubated at 37°C with shaking. After 1 hour the liver digest was filtered through a nybolt filter. This crude extract was processed in the same manner as the rat stellate cell preparation described above. Typically, there would not be sufficient cells to use the elutriator to further purify the cells. Usually a small liver slice (1 x 5 x 4cm approximate dimensions) would yield approximately 20 million human stellate cells of 95% viability and 90-95% purity. Potential contaminating cells include hepatocytes, kupffer cells and sinusoidal endothelial cells.

2.2.3 Culture of hepatic stellate cells.

Quiescent stellate cells were identified by their characteristic autofluoresence because of the presence of vitamin A droplets within the cells. Extracted HSC were cultured on plastic until they were activated to a myofibroblastic phenotype after 7-10 days. Human and rat HSC were used for experiments after activation in primary culture or after passage (<4). Cells were cultured in Dulbecco's modified Eagle's medium (Biowhittaker, UK) in the presence of 16% fetal calf serum and antibiotics penicillin, streptomycin and gentamycin (Gibco, UK). Cells were passaged by first washing three times in HBSS without calcium, then exposing the cells to trypsin in HBSS for 5 minutes at 37°C. The cell suspension was collected, centrifuged gently at 1800rpm for 5 minutes, resuspended in 1ml of fresh medium, and the cells split into new tissue culture flasks.

2.3 Animal models and use of animal materials

2.3.1 Experimental models of progressive fibrosis and fibrosis recovery.

Appropriate home office licence (held by Professor John Iredale) and local ethics committee approval was obtained before work with animals was undertaken. Experimental models of reversible fibrosis and cirrhosis were established by injecting cohorts of 12 Sprague Dawley rats with carbon tetrachloride twice weekly intra peritoneally for 6 and 12 weeks respectively. Control animals were injected with olive oil in parallel. For each model, livers were harvested at peak fibrosis (immediately after the final injection of carbon tetrachloride) and at 5 and 15 days of spontaneous recovery (n=4 at each time point in each model). Harvested livers were split and fixed for haemotoxylin and eosin and Sirius red staining and a portion was snap frozen for biochemical and molecular analysis. Histological analysis of each liver was undertaken and in addition samples of frozen liver at peak fibrosis and 15 days recovery were analysed for hydroxyproline and total collagenase activity as previously described (Iredale et al. 1998). Further sections were cut from each liver, deparaffinised and subjected to microwave antigen retrieval before being immunostained for alpha smooth muscle actin exactly as previously described (Iredale et al. 1998). Three normal untreated rat livers were also harvested for use as controls in individual experiments. The number of alpha smooth muscle actin positive cells was counted by a blinded observer exactly as described previously (Iredale et al. 1998).

2.3.2 Liver homogenisation technique

Rat liver was homogenised using a small piece of whole liver that was previously snap frozen in liquid nitrogen. The fragment of liver was removed from storage at minus 70°C and then kept on liquid nitrogen. Approximately 300mg of liver was weighed out then 0.5ml of ice cold liver homogenisation buffer (see Appendix I) was added. The liver specimen was homogenised for 1 minute using a highspeed rotor (13,000 rpm) that had been autoclaved before. The homogenate was centrifuged at 3000rpm at 4°C for 20 minutes and the 80-100ul supernatant aliquots placed into in labelled tubes. These were then stored at minus 70°C.

2.3.3 Extraction and purification of wild type and mutant r/r collagen.

Acid-soluble type I collagen was extracted and purified from mouse tail using the method previously described (Cawston and Barrett 1979). Collagen was extracted either from wild

type mice or from mice that were homozygous (r/r) for a targeted mutation in the α1 chain which renders the molecule resistant to collagenase cleavage (Kind gift of Dr. S. Krane, Harvard Medical School, Massachusetts General Hospital, Boston, MA, USA)(Krane et al. 1996). The purified collagen was freeze-dried and stored at -20^0C. The purity of the final preparation was checked by SDS PAGE as previously described (Melkko et al. 1990). Extraction of mouse tail collagen was undertaken by Dr Xiaoying Zhou.

2.3.4 Collagen coating of tissue culture flasks

Collagen extracted from mousetail was used to coat tissue plasticware for culturing HSC. Twenty four well tissue culture plates were coated with either wild type or mutant r/r type collagen by incubation with 30μl of 1mg/ml collagen solution in 0.1M acetic acid over night at 4^0C. This was spread evenly over the plate with a sterile cell scraper. The wells were then washed three times with serum free media to remove acid. The HSC were then plated onto the matrix coating and allowed to grow overnight before exposure to experimental conditions.

2.4 DNA methods.

2.4.1 Measurement of DNA concentration by Picogreen technique

Cultured HSC were harvested with a sterile cell scraper into 1ml PBS, pelleted by centrifugation and then resuspended in 500ul TE buffer (10mmol/l Tris-HCL, 1mmol/l EDTA, pH 8.0) before sonication for 15 minutes. 100μl of PicoGreen (Molecular Probes, Eugene, OR) at 1 in 200 dilution was added to 100μl of sample and incubated in the dark at room temperature for 5 minutes. Standards were made from herring sperm DNA. Fluorescence was measured using a Cytofluor II Microwell Fluorescence reader (Perceptive Biosystems, Framingham, MA) at standard wavelengths (excitation 485nm, emission 530nm). Concentrations of double stranded DNA in the samples were calculated from the standard curve.

2.5 RNA methods.

2.5.1 RNA extraction and quantification of quality and concentration

Cultured HSC or whole rat liver homogenate were harvested and total cellular RNA extracted using the Qiagen RNeasy spin column kit (Qiagen, UK). Appropriate precautions were taken to avoid RNA degradation (use of gloves, baked glassware, and filter tips). RNA extracts were quantified using spectrophotometer readings at 260nm (nucleic acids) and 280nm (proteins) (GeneQuant pro, Amersham Pharmacia Biotech). Briefly, 2µl of extracted RNA solution in Rnase free water was used to quantify RNA concentration. This was added to 98µl of Rnase free water. Spectrophotometer reading were taken at 260nm and 280nm and RNA concentration calculated. Purity of the RNA was assessed by the 260nm/280nm ratio (RNA / DNA ratio), a ratio of greater than 1.8 was considered to be satisfactory in purity for the RNA to be used in further analysis. The quality of extracted RNA was assessed by appearance of the 28S and 18S ribosomal RNA bands on RNA integrity gel.

2.5.2 RNA integrity gel

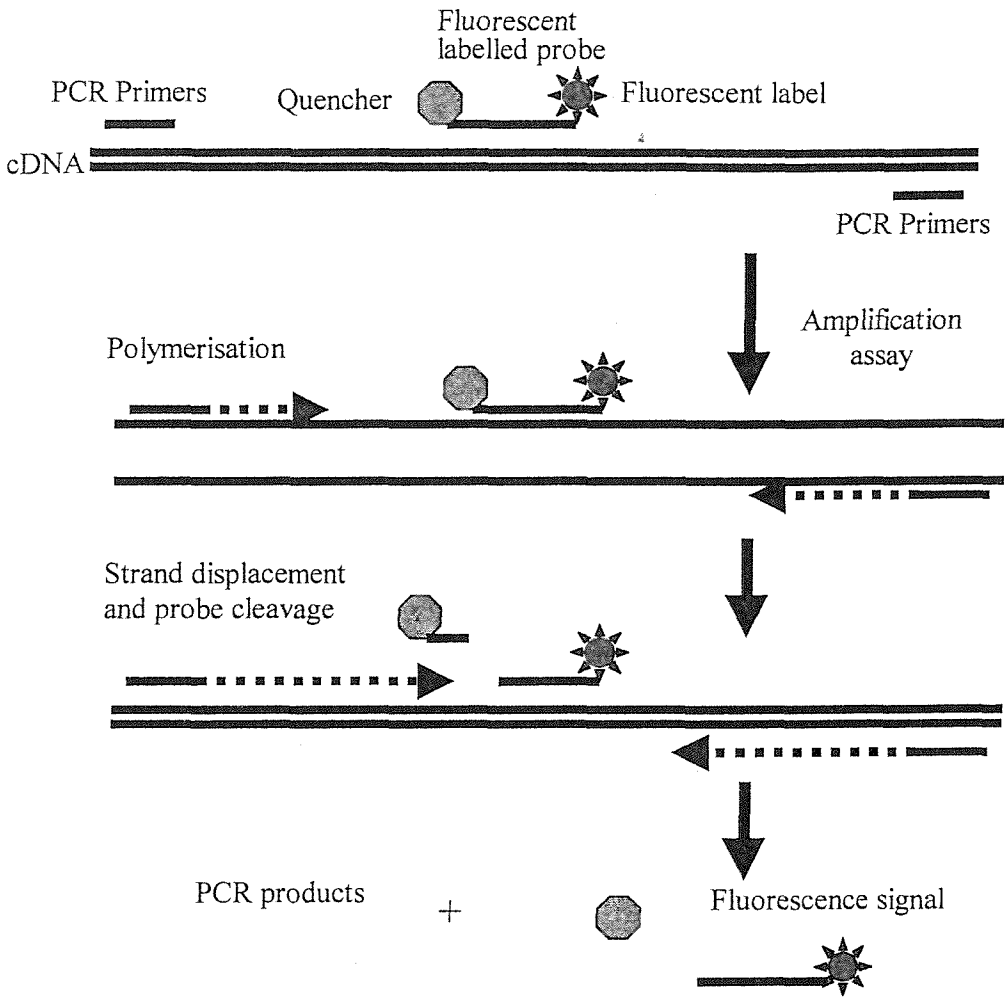
Agarose gel electrophoresis was used to examine the quality and integrity of the extracted RNA. The gel apparatus and comb were assembled and using RNase free baked glassware a 20ml gel was cast after mixing and microwave heating the following reagents: 0.2g agarose, 2ml 10xMOPS, 18ml DEPC water, 1ml formaldehyde (in a fume cupboard). Once the gel had set, it was bathed in RNA running buffer (25ml 10x MOPS and 225ml DEPC water), and RNA samples load carefully (5µl RNA sample and 5µl electrophoresis sample buffer and 1µl of 1mg/ml ethidium bromide). The loaded gel was run at 80-100 volts for 30-40 minutes. The gel was then observed and photographed under UV light to demonstrate the bands of RNA.

2.5.3 Determination of messenger RNA using Taqman realtime quantitative PCR

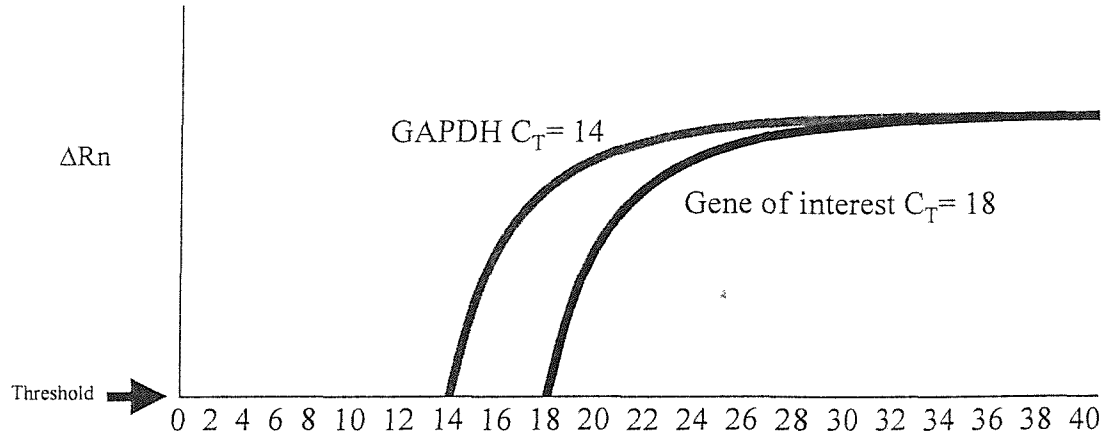
General principles of Taqman PCR technique: The Taqman technique uses primers and probes that are designed to detect a specific target region in the gene of interest. The Taqman probe is labelled with a quenching molecule and a fluorescent molecule. Cleavage of the annealed probe by Taq polymerase results in a loss of quenching, and an increase in fluorescence signal. Normal PCR products are formed leading to an accumulation of cleaved probe during each cycle (see Figure 2.1). After each thermocycle the fluorescence signal

increases and reaches a threshold (ΔRn) that is set to be the same for the gene of interest and the reference gene. The threshold cycle (C_T) is the number of PCR cycles after which there is a detectable fluorescent signal from the reaction tube.

FIGURE 2.1 THE PRINCIPLES OF TAQMAN PCR



2.5.4 Arithmetic formula for relative RNA quantification



The example above will be used to explain relative RNA quantification by Taqman PCR. The threshold cycle (C_T) for the reaction with the primers designed to recognise GAPDH is 14, the threshold cycle for the reaction with the primers designed to recognise the gene of interest is 18. Assuming the PCR reaction for both amplicons occurs with efficiency close to 100%, the amount of target RNA, normalised to the endogenous reference is given by:

$$2^{-\Delta\Delta C_T}$$

Where:

$$\Delta\Delta C_T = {}^{\Delta}C_T \text{ (reference gene) - } {}^{\Delta}C_T \text{(gene of interest)}$$

For example if C_T GAPDH =14 and C_T Gene of interest =18

$$\Delta\Delta C_T = 14 - 18 = -4$$

$$\text{Amount of target relative to GAPDH} = 2^{-(-4)} = 16$$

In the above example there is 16 fold more GAPDH signal compared to the gene of interest. For parallel samples, for example at different time points, the relative differences in the gene of interest from two different RNA samples may be compared by comparing their amount of target relative to GAPDH.

2.5.5 Primer design

All primers and probes were designed using Taqman Primer Express program with the help of Dr Xiaoying Zhou (Postdoctoral fellow), and real time Taqman PCR mRNA quantitation using the PE Applied Biosystems 7700 Sequence Detection System (PE Applied Biosystem, Warrington Cheshire, UK). The following rules for primer design are employed in the Primer Express program:

Probe checklist:

1. Amplicon size of 50-150 base pairs.
2. Primer and probe sequences correctly match the original amplicon sequence and are in the correct 5'-3' orientation.
3. No G on the 5' end.
4. Percentage GC in the range of 20-80%
5. No runs of more than 3 consecutive Gs
6. Primer Express Tm of 68-70°C for a single probe application.

Primer checklist:

1. Forward and reverse primers are as close as possible to the probe without overlapping probe.
2. Percentage GC in the range 20-80%.
3. No runs of more than 3 consecutive Gs.
4. Primer Express Tm of 58-60 °C.
5. Five nucleotides at the 3' end have only 1-2 G+C.

Primers and probe sequences of Rat GAPDH used were:

sense: 5'–ggcctacatggcctccaa- 3',

anti-sense: 5'-tctctcttgctctcagtatccttgc-3'

probe: 5'-agaaaccctggaccacccagccc-3'

Rat TIMP-1 primers and probe sequences used were:

sense: 5'-agcctgtagctgtgccccaa-3',

anti-sense: 5'-aactcctcgctgcggttctg-3'

probe: 5'-agaggctctccatggctggggtgta-3'.

2.5.6 Preparation of cDNA from total RNA

Total RNA from liver homogenate or cultured HSC was extracted using the RNeasy kit (Promega, UK). The first strand cDNA synthesis was undertaken using random primers and moloney murine leukaemia virus reverse transcriptase system (Promega, Southampton, UK). 1 μl of first strand cDNA (10 ng RNA) and 0.3 μM of primers and 0.3 μM of probe were used in per 25 μl Real-time Taqman PCR reaction. Taqman 2x Universal PCR Master Mix and 0.2 ml optical Reaction tube (PE Applied Biosystem, Warrington, Cheshire, UK) were employed. Appropriate negative controls were included. These were samples without cDNA and also sample of RNA subjected to the cDNA synthesis step without reverse transcriptase present. The conditions of the reaction were as follows: initial steps were 50 ^{0}C, 2 min and 95 ^{0}C 10 min, followed with a denaturing step for 15 sec at 95 ^{0} C, and an annealing extension step at 60 ^{0} C for 1 min. Determination of the expression of the house keeping gene, GAPDH was employed and all reactions were undertaken in triplicate. After detection of the threshold cycle for each mRNA in each sample, relative concentrations were calculated and normalised to GAPDH analysed in parallel.

2.5.7 Gene array analysis

Total cellular RNA extracted from cultured human activated HSC was used to assess the effect of TIMP-1 and the mutant T2G TIMP-1 on apoptosis related genes. The human apoptosis GEArray Q series (Superarray Kit, Cambridge Bioscience, UK) was used to screen for target gene that might help explain the anti apoptotic effect of TIMP-1. This array consists of a panel of 96 key genes involved in apoptosis. Genes are grouped into nine categories according to their functional and structural features. These included:

Tumour necrosis factor ligand family	14 genes
Tumour necrosis factor receptor family	15 genes
Bcl-2 family	14 genes
Caspase family	12 genes
Inhibitors of apoptosis (IAP) family	6 genes
TNF receptor associated factor (TRAF) family	8 genes
CARD family	6 genes
Death domain family	5 genes
Death effector domain family	4 genes
CIDE domain family	4 genes
P53 and ATM pathway	10 genes
House keeping genes and bacterial negative control	5 genes

This array was used following the manufacturer's instructions. Briefly, 5μg of total cellular RNA was annealed to gene specific primers provided in the kit. This was converted to cDNA probes by reverse transcription and labelling with Biotin dUTP (Roche, UK). The probes were allowed to hybridise overnight with the gene array. Chemiluminescence detection was used and X-ray films taken of varying times of exposure. Scanned images were quantified using ImageQuant software. X-Ray images were scanned as a 16 bit grayscale image and saved as TIFF files. An eight by fourteen grid was laid over the image and a raw data report generated by Image Quant. The X-Ray image is a negative, therefore the data report values were inverted following the software instructions by using the following equation:

Inverted data value = 65536 – Data volume report

For example, Volume report for GAPDH = 6536
Then the Inverted GAPDH value = 65536 – 6536 = 59000

The array includes an important negative control pUC 18 which is from a bacterial plasmid. The volume report from this part of the array was considered to represent zero. Inverted data were converted into arbitrary percentages with the value of pUC 18 negative control assigned 0 % and GAPDH assigned 100%. Given the arbitrary nature of quantification of the array imaging software, the conclusions regarding changes in gene expression must be made with great care. To safeguard for this, we adopted a pragmatic approach where only spots that were visible to the naked eye were quantified and then expressed as fold changes (no change, 2-10 fold change and greater than 10 fold change). Candidate genes where selected for further study at the protein expression level either by ELISA or western blotting.

2.6 Protein methods.

2.6.1 Measurement of protein concentration in cell extracts.

The protein concentration of cell extracts was performed using a protein dye binding kit (D_c Protein assay kit, BioRad, USA). Briefly, 2µl of protein extract was added to 100µl of reagent A and 800µl of reagent B and this was left for 15 minutes at room temperature. Parallel standards were made using BSA (0-20mg/ml). Absorbance was measured at 750nm on a spectrophotometer (Ultrospec 2100 pro UV/Visible spectrophotometer). A standard curve was plotted and the protein extract protein concentrations calculated from the standard curve equation. The standard curve equation was formed by a linear regression using the Prism software package.

2.6.2 Western blotting technique

Western blot analysis of rat liver tissue and HSC protein extracts were undertaken using a monoclonal anti alpha smooth muscle action (α-SMA) antibody (Sigma, Poole, UK) and an antibody to Bcl-2 and Bax (rabbit polyclonal, clones sc-492 and sc-520 respectively), N-Cadherin extracellular domain ECD1 (Clone 3B9, Zymed, Santa Cruz, California) and N-

Cadherin cytoplasmic domain (Clone CG-4, Sigma, UK) to detect protein expression. The extracted proteins were subjected to electrophoresis on 8% SDS-PAGE gel after normalisation for protein content. After resolution, the protein samples were electrotransferred onto PVDF in transfer buffer (see appendix I). The membrane was blocked for 1 hour in 5% non-fat dry milk in TBS. Membranes were incubated overnight at room temperature with the primary antibody (1:500) or with non-immune IgG (as negative control) in TBS. Membranes were washed 3 times for 15 minutes in 0.1% Tween TBS before the addition of the secondary antibody (rabbit anti-mouse IgG HRP in a dilution 1:2000) in TBS containing 0.5% non-fat dry milk for 1 hour. The membranes were then washed in TTBS twice for 10 min and followed by distilled water for 10 min. Reactive bands were identified using enhanced chemiluminescence (ECL, Amersham, UK) and autoradiography according to the manufacturer's instructions.

2.6.3 ELISA for Fas and Fas ligand

Human HSC were grown to confluence and exposed to BSA with and without TIMP-1 (5nM) overnight. Cells and supernatants were harvested and protein extracts assayed for Fas and Fas ligand by commercial ELISA (Calbiochem, Nottingham, UK) following the manufacturer's instructions. Briefly, human cell samples were assayed and compared against recombinant standards. Quantity of Fas and Fas ligand were normalised to cell number by DNA quantification using the Picogreen technique.

2.6.4 Gelatin zymography of HSC conditioned media and rat liver extracts

Extracts from in vitro apoptosis experiments and rat liver from experimental models of liver fibrosis were analysed for MMP-2 by gelatin zymography(Benyon et al. 1996). Briefly, 30μg of whole liver homogenate or 10μl of HSC conditioned media was resolved on 8% SDS-PAGE gels. The SDS was washed out of the gel by three 10 minute washes in 2.5% Triton X-100. The gel was incubated in MMP proteolysis buffer (50mM Tris/HCl pH 7.8, 50mM $CaCl_2$, 0.5M NaCl) overnight at 37°C. The gels were rinsed in water and then stained with Coomassie blue (0.15% Coomassie blue, 10% Acetic acid, 40% Methanol, 50% water) for 1 hour. The gels were destained using 7.5% acetic acid, 10% methanol, 82.5% water. Pro and active forms of MMP-2 were compared against standard molecular weight markers (Amersham, UK).

2.6.5. Measurement of active MMP-2 by Biotrak activity assay system

The level of active MMP-2 in supernatants from cultured human HSC was quantified using the Biotrak MMP-2 activity assay (Amersham, UK). The assay uses the pro form of a detection enzyme (urokinase) that can be activated by captured active MMP-2, into an active detection enzyme through a single proteolytic event. The natural activation sequence of the pro detection enzyme has been replaced using protein engineering, with an artificial sequence recognised specifically by active human MMP-2. The active detection enzyme can then be measured using a specific chromogenic peptide substrate. Using standards of human pro MMP-2 that are activated by exposure to p-aminophenylmercuric acetate (APMA), samples may be assayed in a simple ELISA protocol. Samples of supernatants from cultured human HSC were assayed for active MMP-2 in this manner following the manufacturer's instructions.

2.6.6. Supply of wild type and T2G mutant N-TIMP-1 proteins

As a collaboration for this project between Professor John Iredale and Professor Hideaki Nagase of Imperial College London and Professor Keith Brew of Miami School of Medicine, wild type and T2G mutant N-TIMP-1 was supplied to our laboratory. The protein was manufactured by Dr Q Meng who was a PhD student in the department of molecular biology and biochemistry of Miami School of Medicine. The mutant proteins were made using a PCR primer based technique, ligation into an expression vector, and then transfection of E. Coli bacteria. The mutant protein was extracted and purified and its structure studied by CD spectroscopy and 2D NMR (Huang et al. 1996). N-TIMP-1 differs from full size TIMP-1 which contains both N and C terminal domains. N-TIMP-1 binds with a 6-8 fold less affinity to MMPs 1, 2, and 3 when compared to full size TIMP-1. The T2G mutant N-TIMP-1 has a reduced affinity for MMP-1 and MMP-2 by greater than three orders of magnitude rendering it ineffective as an inhibitor of active MMPs (Meng et al. 1999; Nagase et al. 1999).

2.6.6 Immunofluorescence confocal laser microscopy for N-Cadherin and beta catenin.

Human HSCs were grown on sterilised round glass coverslips (Chance, UK). Cultured cells were subjected to a range of conditions. These included 16% serum, serum deprivation,

gliotoxin (1.5μM), in the presence or absence of active MMP-2 (10nM) (CN Biosciences, UK), MMP2-inhibitor I (17μM, 1.7μM, 0.17μM) (Calbiochem, UK), recombinant human TIMP-1 (10nM) (CN Biosciences, UK) and recombinant human TIMP-2 (10nM) (CN Biosciences, UK). Following a 4 hour incubation at 37°C in 5% CO_2, the treatment media was removed and the glass coverslips were washed in PBS. The glass coverslips were fixed with acetone for 10 minutes, washed 3 times for 5 minutes in PBS. Primary antibodies for N-cadherin (Dilution 1:50, Clone 3B9, Zymed laboratories, USA and clone GC-4, Sigma, UK) and β-catenin (Dilution 1:75, Transduction laboratories) were applied in PBS. Primary antibody was placed onto each glass coverslip and incubated at room temperature for 1 hour. After removing the primary antibody, the glass coverslips were washed 3 times for 5 minutes with PBS solution, before being incubated for 30 minutes with the secondary antibody, Mouse Immunoglobulin FITC (Dako) in 1% BSA PBS solution. After a subsequent wash in 0.01M PBS for 5 minutes, the glass coverslips were counter stained for 10 minutes with 7-amino actinomycin D (Sigma, UK) in 1% BSA PBS solution. The glass coverslips were finally mounted on glass slides using mowiol and observed under a Leica SP-2 confocal laser scanning microscope.

2.7 Stimulation and quantification of apoptosis.

2.7.1 Overview of quantification of apoptosis:

There are numerous methods measure apoptosis (Hall 1999). The phenomenon of apoptosis is defined by a series of morphological changes (Kerr et al. 1972). The characteristic features are best observed by electron microscopy but can be observed at the light microscope using nucleic acid binding dyes, such as haematoxylin, acridine orange, or propidium iodide (Coles et al. 1993; Hall et al. 1994). There has been a large increase in the volume of work in the field of apoptosis. This has led to a proliferation in the number of available methods to quantify apoptosis. The biochemistry of apoptosis is now known in some detail. Many methods use one specific part of the apoptosis pathway as a surrogate marker of apoptosis (Figure 1.9). Annexin V expression on the cell surface is thought to be an early event in apoptotic cell. Caspase-3 activation is considered an intermediate step in apoptosis. DNA fragmentation is thought to be a late manifestation of apoptosis. A generic problem with apoptosis measurement is that for a given population of cultured cells exposed to an apoptotic stimulus at the same time, the lead in or lag time before morphologic features of apoptosis

occur is variable. Once apoptosis has started, it occurs in an efficient manner and some reports claim that in as little as 1-2 hours a cell can undergo apoptosis and the apoptotic cell debris can be removed by neighbouring cell phagocytosis (Coles et al. 1993). To attempt to measure apoptosis by a single method is therefore prone to error. Measurement of apoptosis usually involves static measurement and so it is incorrect to discuss 'apoptotic rates'. For this reason, morphological techniques are normally expressed as an apoptotic index or percentage. Given the static nature of methods employed to quantify apoptosis, most would accept that at least two or three different methods are required to verify if a given change is real. For this reason both morphologic and biochemical means for quantifying apoptosis have been used in this work. Each method has its advantages and disadvantages. Morphological techniques are technically relatively easy and sensitive, but are prone to subjective bias of the observer (Hall 1999). Biochemical methods to quantify apoptosis can be less sensitive because some of the apoptosis products are relatively short lived (for example active Caspase-3 enzyme) therefore the timing of cell harvest and sample handling are critical for biochemical methods to work well (Kohler et al. 2002).

2.7.2 Examination of nuclear morphology by Acridine Orange

Apoptosis of HSC was always undetaken in serum free conditions with of with out proapoptotic stimuli as follows. Apoptosis of HSC was induced by absolute serum deprivation (greater than 18 hours), cycloheximide (50μM for 4 hours) treatment (Issa and Williams 2001), exposure to nerve growth factor (100ng/ml for 4 hours) or gliotoxin exposure (0.375-1.5μM for 4 hours) as previously described (Trim et al. 2000; Wright et al. 2001). HSC were cultured in 24 well tissue culture plates. Rat and human HSC were exposed to pro apoptotic stimuli with and without recombinant TIMP-1 and TIMP-2 (Calbiochem, U.K.) and other manipulations as detailed below. Following a 4 hour incubation at 37°C, nuclear morphology was assessed by adding 2μl of 1mg/ml Acridine Orange (Sigma, UK) to each well (final concentration 1 ug/ml) and observing the cells under blue fluorescence (Leica, Inverted fluorescence microscope). The total number of apoptotic bodies were counted and any apoptotic bodies floating in the supernatant included by racking up the objective lens. The total number of cells per field was counted and an apoptotic index calculated. Each condition was performed in duplicate and three high power fields were counted for each well. Experiments were repeated in parallel following an 18 hour incubation in serum free

conditions. To examine for autocrine effects, HSCs were incubated for 18 hours with azide free polyclonal neutralising antibodies to TIMP-1 (Clone sc-6834), TIMP-2 (Clone sc-5539), non immune IgG control (Santa Cruz Biotechnology, California) and responses assessed by Acridine Orange staining and counting. Parallel experiments using the non functional T2G mutant N-TIMP-1 and wild type TIMP-1 proteins (kind gift of Professor Hideaki Nagase, Imperial College, UK and Professor Keith Brew, Department of Molecular Biology, Miami School of Medicine, USA) were performed in which apoptosis was induced by cycloheximide and assessed by the acridine orange technique.

2.7.3 Quantification of apoptosis by Caspase-3 activity assay

To support the data from Acridine Orange counting, experiments with recombinant TIMP-1, TIMP-2, the inactive T2G mutant N-TIMP-1, the wild type TIMP-1 proteins and the broad spectrum caspase inhibitor zVAD-fmk were repeated and apoptosis quantified by a colorimetric assay for Caspase-3 activity (Calbiochem, Nottingham, UK) according to the manufacturer's instructions. This kit uses a colorimetric substrate to active Caspase-3 that yields p-nitroanaline (pNA) which is yellow in colour. The change in absorption at wavelength 405nm is proportional to the caspase-3 activity in the protein extracts. Typically a T-75 flask was required for each experimental condition. Cells were collected by cell scraping into 1ml sterile PBS, pelleted and resuspended in a cell lysis buffer supplied with the kit (50mM HEPES,1mM DTT, 0.1mM EDTA, 0.1% CHAPS, pH 7.4). Cells were lysed at 4°C on ice for 5 minutes then centrifuged at 10,000g for 10 minutes at 4°C. The protein concentration was determined by the coomasie blue technique described below. Typically 30μg of protein extract was used in each reaction. A positive and negative control was included by way of recombinant active Caspase-3 and z-VAD-fmk (broad spectrum caspase inhibitor) respectively. Caspase-3 activity is either expressed as change in absorption after 4 hours incubation or a dynamic study is shown with multiple absorbance readings taken every 15-20 minutes over 4 hours. Dynamic data were used in a linear regression to give caspase-3 activity in pmol pNA/hour/μg protein extract. To determine if TIMP-1 or TIMP-2 directly inhibited activity of Caspase-3, each recombinant protein was incubated with recombinant Caspase-3 for 1 hour at 37 °C before adding the Caspase-3 substrate and then Caspase-3 activity measured as described above.

2.7.4 Quantification of apoptosis by TUNEL staining

To verify observation made with acridine orange staining with cell counting and caspase-3 activity assays, HSC were cultured on glass chamber slides and then exposed to 50μM cycloheximide for 18 hours with and without TIMP-1 and TIMP-2 (100ng/ml). Slides were then stained for DNA fragmentation characteristic of apoptosis by the TUNEL reaction as previously described (Iredale et al. 1998) with the modifications recently described to reduce false positivity (Stahelin et al. 1998). Each slide was then analysed by a blinded observer who counted the number TUNEL positive apoptotic figures and the TUNEL negative cells over ten high power fields for each condition.

2.8 Quantification of cellular proliferation methods.

2.8.1 Cell proliferation assay: Effect of TIMP-1, TIMP-2 and MMP-2 on HSC proliferation

HSC were cultured in 24 well tissue culture plates. These were washed with serum free media for 24 hours then the cells were exposed to TIMP-1 and TIMP-2 at a concentration range of 1-100ng/ml or active MMP-2 for 24 hours and then pulsed with tritiated thymidine (0.5uCi per well) for 18 hours. Appropriate care was taken using tritiated thymidine. After washing the cells three times with HBSS with calcium the cells were fixed using pre cooled 95% methanol and 5% acetic acid in the freezer at -20°C for 30 minutes. Next the cells were washed again three times with HBSS with calcium. The cells were then lysed with cell dissolution solution (0.25M NaOH and 0.02% SDS) for 15 minutes on a shaker. Next 5M HCl acid was added to each well to neutralise the NaOH. The well contents were transferred to mini vials, 3ml of scintillation fluid was added. Scintillation counting was undertaken at 2 minutes per sample (Boulton and Hodgson 1995). Data were expressed as mean and standard error counts per minute for each experimental condition. Experiments were repeated using HSC extracted from separated rat HSC or human HSC preparations.

2.9 Statistical analysis.

Statistical analysis was undertaken using the Graphpad Prism statistical package (Graphpad software Incorporated, USA). Most experiments were repeated each with separate cell preparations between three to six times. This meant that most experiments were repeated with three to six separate isolations of HSC from different animals of human liver specimens from different patients. Acridine orange counting experiments used duplicate wells for each experimental condition and three random counts were taken from each well, for example a single condition in each experiment would give six apoptotic indices and if this was repeated three times with differents cells, there would be eighteen indices to use in the data analysis per experimental condition. Caspase-3 activity assays were undertaken using single flask (T-75 size) and triplicate wells were used for each experimental condition. TUNEL staining and counting used twenty counts per experimental condition. Unless otherwise stated, differences of means were assessed using the Student's t test. Differences were considered statistically significant if $p<0.05$ (*), and very significant if $p<0.005$ (**).

Chapter 3

3. Studies of TIMP-1 and TIMP-2 effects on hepatic stellate cell survival

3.1 Introduction

3.1.1 Liver fibrosis represents the final common pathological outcome for the majority of chronic liver insults (for example alcohol, autoimmune or viral injury)(Alcolado et al. 1997). Current evidence indicates that the central mediator of liver fibrosis is the hepatic stellate cell (Friedman 1993). During fibrotic injury, these retinoid rich perisinusoidal cells proliferate and undergo a phenotypic transformation to myofibroblast-like cells, a process termed activation (Bachem et al. 1992; Friedman et al. 1985). Previous work has demonstrated that in addition to collagen-I (Maher and McGuire 1990), activated HSC also express tissue inhibitors of metalloproteinases (TIMPs) 1 and 2, leading to the hypothesis that matrix degradation is inhibited during progressive fibrosis (Arthur et al. 1989; Benyon et al. 1996; Herbst et al. 1991; Iredale et al. 1996; Milani et al. 1992; Vyas et al. 1995; Winwood et al. 1995). This hypothesis is supported by findings that over expression of TIMP-1 enhances experimental fibrosis (Yoshiji et al. 2000) and that spontaneous recovery from liver fibrosis is associated with a diminution of TIMP expression and an increase in collagenase activity with consequent matrix degradation (Iredale et al. 1998). A further finding in this study was that apoptosis was responsible for mediating HSC loss during recovery from fibrosis (Gong et al. 1998; Iredale et al. 1998; Issa et al. 2001; Saile et al. 1997). This has highlighted the control of HSC apoptosis as a key process regulating fibrosis in toto. Indeed, it has recently been demonstrated that induction of HSC apoptosis has an anti fibrotic effect (Wright et al. 2001).

3.1.2 Whilst previous work has emphasised the potential importance of TIMPs to fibrosis via inhibition of matrix degradation, individual TIMPs may regulate cell division and apoptosis independently of this activity. TIMP-1 has been shown in vitro to suppress apoptosis of Burkitt's lymphoma cell lines (Guedez et al. 1998) and human breast epithelial cells (Li et al. 1999). In these studies the anti apoptotic effect of TIMP-1 was reported to be independent of its ability to inhibit MMP activity. Moreover, several neoplasms demonstrate a positive correlation between TIMP-1 and TIMP-2 expression and metastatic spread. This observation is counter intuitive to the previously accepted hypothesis that TIMP-1 mediated inhibition of matrix degradation would retard tumour dissemination (Ree et al. 1997). TIMP-1 was originally identified as a growth factor for myeloid elements and has also been demonstrated

to promote fibroblast proliferation (Docherty et al. 1985, Kikuchi et al. 1997). These data suggest that TIMPs may be important regulators of cell growth and apoptosis.

3.1.3 The TIMPs appear to have divergent effects on proliferation and apoptosis in different cell types. For example TIMP-2 acts as a growth factor for mesenchymal cells in rat kidney development (Barasch et al. 1999) while it is a pro apoptotic stimulus for human T lymphocytes (Lim et al. 1999). Adenoviral over expression of TIMP-1, -2 and -3 has been studied in rat aorta smooth muscle cells. Within the same cell type the TIMPs had divergent effects, TIMP-1 over expression had no effect on cell proliferation while TIMP-2 produced a dose dependent reduction in proliferation. This effect was not mimicked by a synthetic matrix metalloproteinase inhibitor. TIMP-3 over expression induced DNA synthesis and promoted apoptosis in myofibroblasts (Baker et al. 1998). In contrast, TIMP-2 over-expression in B16F10 melanoma cells protects these cells from apoptosis but had no effect on proliferation (Valente et al. 1998). The close correlation between the reduction of TIMP-1 and TIMP-2 expression and apoptosis of HSC observed in vivo during recovery from liver fibrosis highlights a possible role for TIMP-1 and TIMP-2 in regulating HSC survival.

3.2 In vitro studies of TIMP-1 and TIMP-2

3.2.1 TIMP-1 and TIMP-2 inhibit apoptosis induced by cycloheximide, serum deprivation & nerve growth factor

Assessment of nuclear morphology following acridine orange staining was undertaken (Figure 3.1). Time course studies of HSC induced into apoptosis by cycloheximide demonstrated that after 4-6 hours exposure to cycloheximide there was a significant number of HSC showing apoptotic morphology (Figure 3.2). Further studies showed that 4 hours incubation with TIMP-1 or TIMP-2 significantly reduced apoptosis of rat and human HSC induced by cycloheximide in a dose dependent manner at a concentration range of 1-200ng/ml (figure 3.3a & 3.3b, 3.4a & 3.4b respectively). An identical effect with TIMP-1 and TIMP-2 was observed after 24 hours incubation in serum free conditions (data not shown). In each experimental condition TIMP-1 was found to be more effective at inhibiting apoptosis than TIMP-2 on a per nM basis. Bovine serum albumin, used as a carrier for the TIMPs had no anti-apoptotic effect.

FIGURE 3.1 ACRIDINE ORANGE STAINING OF CULTURED HEPATIC STELLATE CELLS

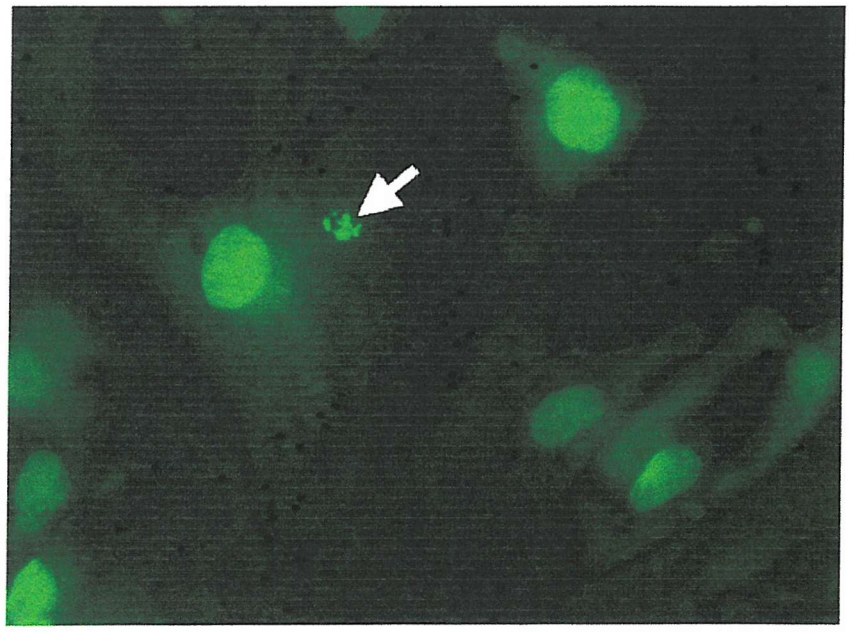


Figure 3.1 Example of an apoptotic HSC (arrowed) induced by cycloheximide exposure for 4 hours and identified in situ by acridine orange staining. A normal cell lies adjacent to the apoptotic body.

FIGURE 3.2. TIME COURSE OF HSC DEMONSTRATING APOPTOTIC MORPHOLOGY AFTER EXPOSURE TO CYCLOHEXIMIDE AND SERUM DEPRIVATION

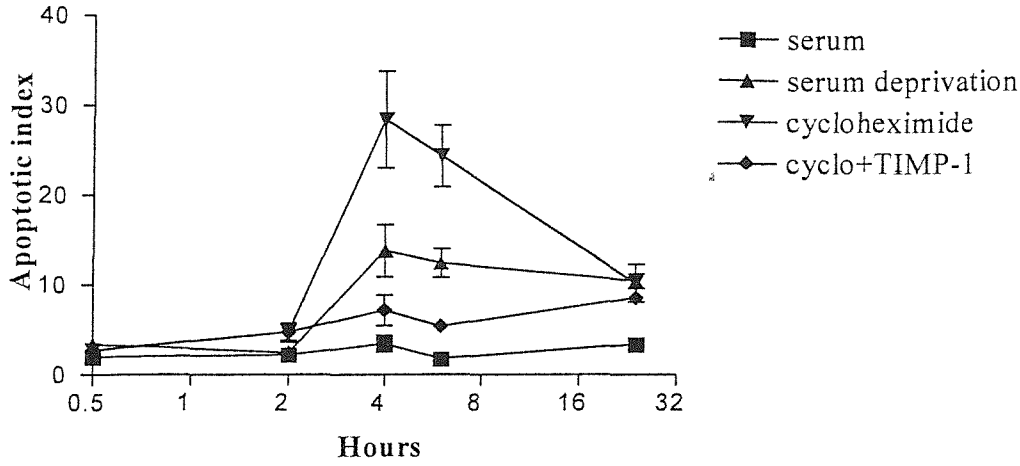


Figure 3.2. A time course study was undertaken to determine the earliest time activated HSC showed apoptotic morphology after a pro apoptotic stimulus. In this case cells were exposed to 50μM cycloheximide or absolute serum deprivation. After 4-6 hours, cycloheximide induced a significant number of rat HSCs to enter apoptosis. However, this effect was not sustained and after 24 hours its effect reduced back to a level comparable to serum deprivation alone. Cells treated with cycloheximide and recombinant human TIMP-1 (200ng/ml) appeared to be resistant to enter apoptosis. Data are mean and SEM, n=3.

FIGURE 3.3A. EFFECT OF RECOMBINANT TIMP-1 ON RAT HSC INDUCED INTO APOPTOSIS BY CYCLOHEXIMIDE

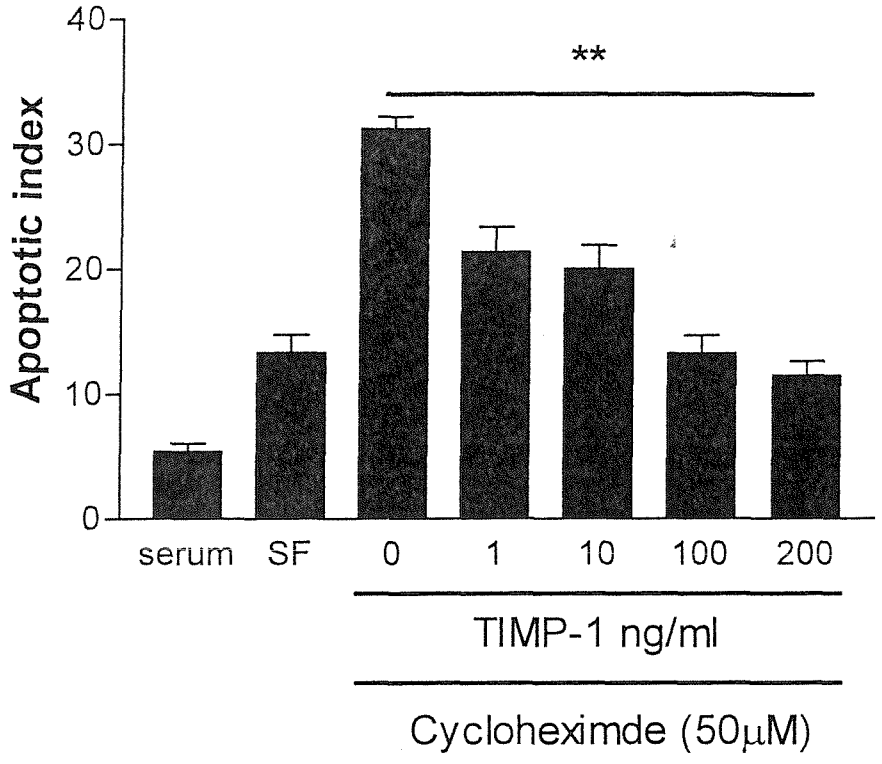


Figure 3.3a. TIMP-1 significantly reduced apoptosis of activated rat hepatic stellate cells induced by 4 hours cycloheximide exposure in a dose dependent manner over the concentration range 1-200ng/ml (0.035-7nM). Apoptosis determined by acridine orange staining and counting. (Data presented are mean +/-SEM apoptotic index expressed as percentage of total cells counted. **$p<0.001$ for cycloheximide versus cycloheximide with 200ng/ml TIMP-1 by Student's t-test, n=5)

FIGURE 3.3B EFFECT OF TIMP-1 ON HUMAN HSC INDUCED INTO APOPTOSIS BY CYCLOHEXIMIDE

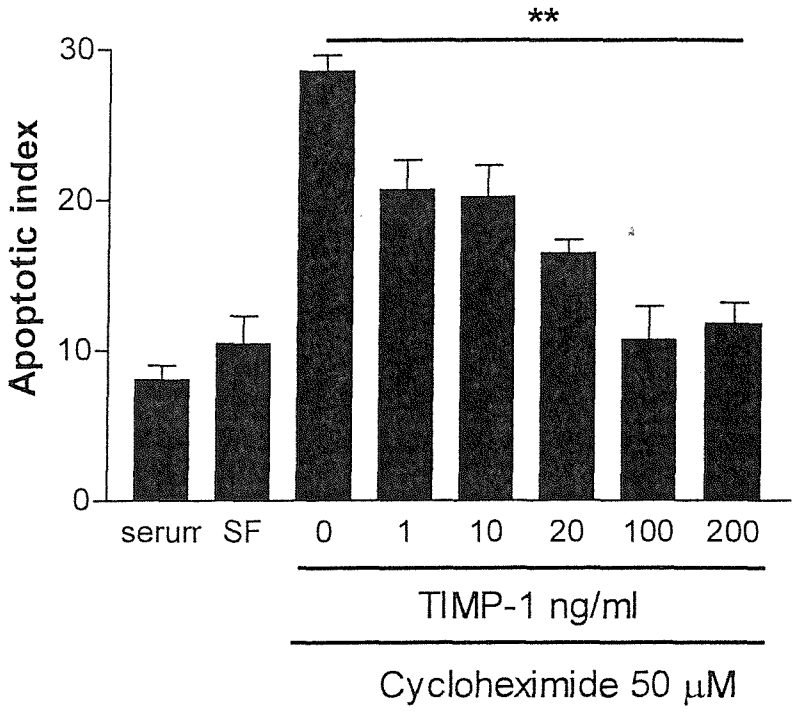


Figure 3.8b. TIMP-1 significantly reduced apoptosis of activated human hepatic stellate cells induced by 4 hours cycloheximide exposure in a dose dependent manner over the concentration range 1-200ng/ml (0.035-7nM). Apoptosis determined by acridine orange staining and counting. (Data presented are mean +/-SEM apoptotic index. **p<0.005 for cycloheximide versus cycloheximide with 200ng/ml TIMP-1 by student's T-test, n=5)

FIGURE 3.4A. EFFECT OF RECOMBINANT TIMP-2 ON ACTIVATED RAT HSC APOPTOSIS INDUCED BY CYCLOHEXIMIDE.

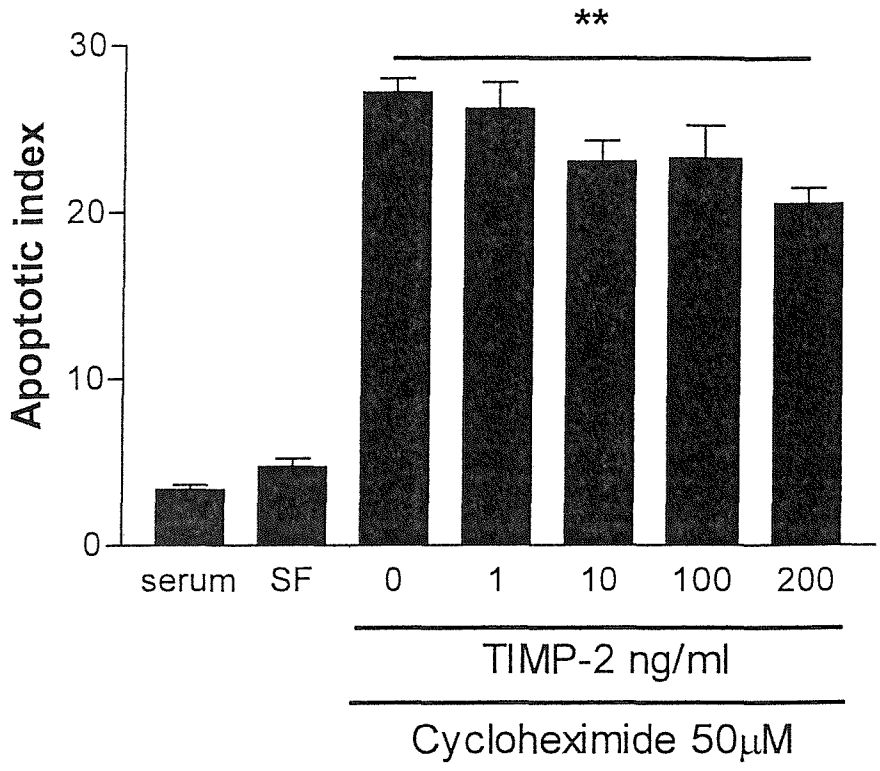


Figure 3.4a. TIMP-2 significantly inhibits apoptosis of activated rat stellate cells induced by 4 hours exposure to cycloheximide at a concentration range of 1-200ng/ml (0.048-9.5nM). (Data expressed are mean +/- SEM apoptotic index. **p<0.001 for cycloheximide alone versus cycloheximide and 200ng/ml TIMP-2 by student's T-test, n=6).

FIGURE 3.4B EFFECT OF TIMP-2 ON HUMAN HSC INDUCED INTO APOPTOSIS BY CYCLOHEXIMIDE

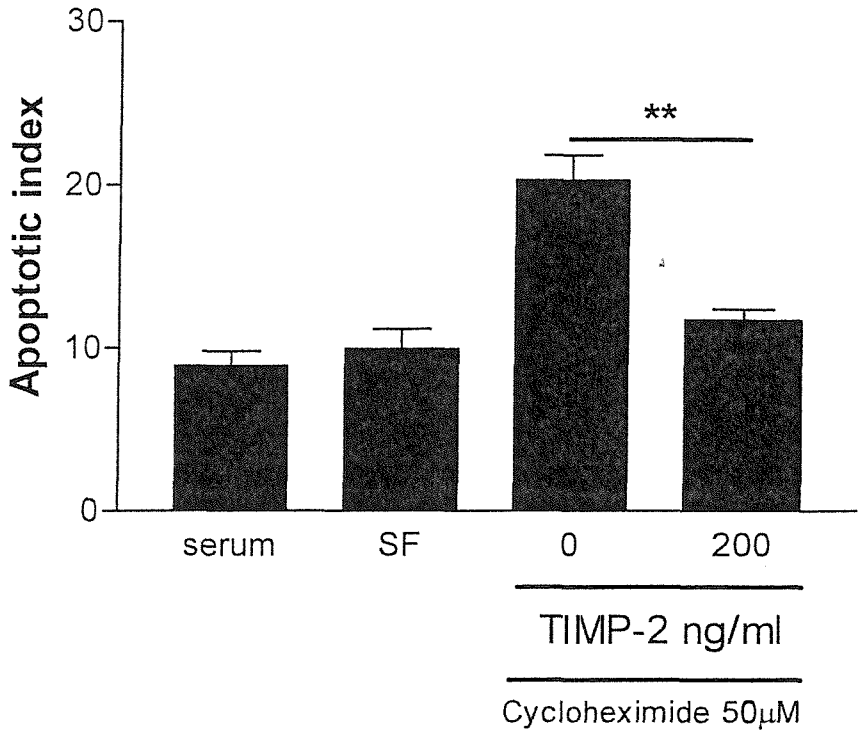


Figure 3.4b. TIMP-2 significantly inhibits apoptosis of activated human stellate cells induced by 4 hours exposure to cycloheximide at a concentration of 200ng/ml (9.5nM). (Data expressed are mean +/- SEM apoptotic index. **p<0.005 for cycloheximide alone versus cycloheximide and 200ng/ml TIMP-2 by Student's t-test, n=3).

3.2.2 Validation of the Acridine orange staining experiments

These early experiments with recombinant TIMP-1 and TIMP-2 showed exciting results. To validate the apparent anti apoptotic effect of the TIMPs on rat HSC experiments were repeated using a different techniques to quantify apoptosis. These were the Caspase-3 activity assay and TUNEL staining and counting. The Caspase-3 experiments demonstrated a similar anti apoptotic effect with both TIMPs. Furthermore, the effect of TIMP-2 was less than that observed for TIMP-1 (Figure 3.5). This demonstrated that the acridine orange staining and counting was a valid and robust method to quantify apoptosis in cultured HSC. This simple technique was therefore used extensively in this work.

3.2.3 TIMP-1 and TIMP-2 treated HSC have reduced Caspase-3 activity following induction of apoptosis by cycloheximide.

Caspase-3 is a central caspase in the pro apoptotic cascade (Hengartner 2000) and can be used as an alternative assay to assess apoptosis. HSC cultured in 50μM cycloheximide with TIMP-1 or TIMP-2 (1-100ng/ml) demonstrated a dose dependent reduction in Caspase -3 activity compared to cycloheximide alone (Figure 3.10).

FIGURE 3.5. CASPASE-3 ACTIVITY OF ACTIVATED HSC INDUCED INTO APOPTOSIS BY CYCLOHEXIMIDE AND EFFECTS OF TIMP-1 AND TIMP-2

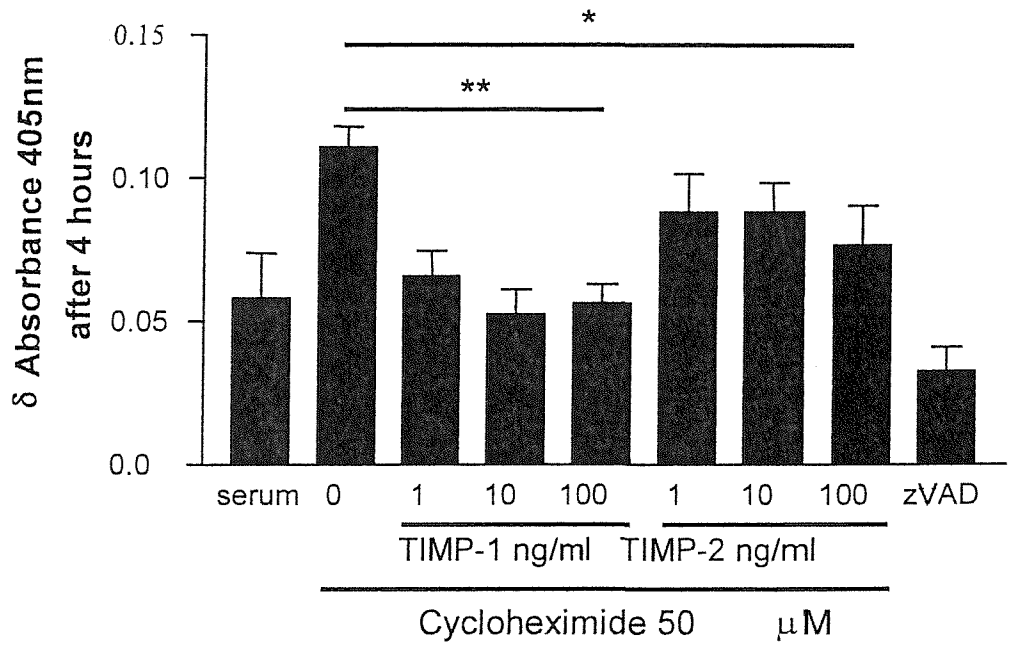


Figure 3.5. Caspase-3 activity of cell extracts from rat HSC incubated for four hours with cycloheximide, with and without TIMP-1 and TIMP-2 were compared to HSC incubated in serum and zVAD-fmk (a broad spectrum caspase inhibitor). TIMP-1 and to a lesser extent TIMP-2 show a dose dependent reduction in caspase-3 activity over the concentration range 1-100ng/ml. (Data are expressed as mean +/- SEM change in absorbance after four hours of incubation in the caspase assay; *$p<0.05$; **$p<0.005$; n=3)

3.2.4 TIMP-1 treated HSC have reduced DNA fragmentation.

A further pathognomonic feature of apoptosis is the fragmentation of DNA into oligonucleosomal lengths (Evan et al. 1992). Fragmented DNA can be identified by the TUNEL technique, which can therefore be used to further quantify the apoptotic response of HSC in the presence and absence of cycloheximide. Activated HSC cultured on glass chamber slides and exposed to cycloheximide for 18 hours with TIMP-1 and TIMP-2 demonstrated significantly reduced numbers of cells containing fragmented DNA assessed by the TUNEL technique compared to controls treated without TIMPs (Figure 3.6).

FIGURE 3.6 EFFECT OF TIMP-1 AND TIMP-2 ON DNA FRAGMENTATION.

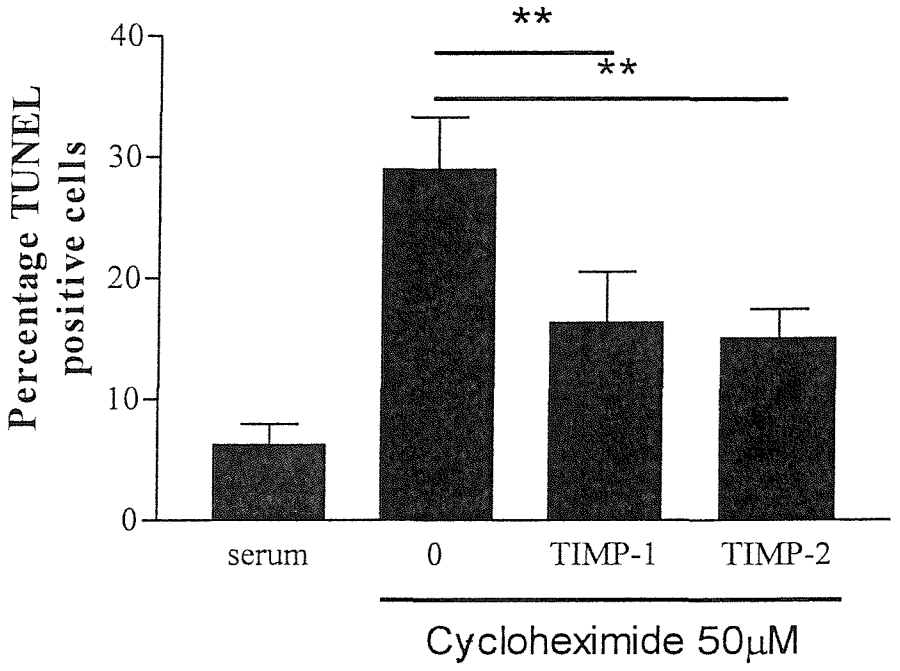


Figure 3.11. Rat activated hepatic stellate cells were induced to undergo apoptosis by cycloheximide treatment for eighteen hours in the presence and absence of TIMP-1. Treatment with TIMP-1 or TIMP-2 (100ng/ml) resulted in significantly reduced numbers of apoptotic cells defined by the presence of DNA fragmentation detected by the TUNEL technique. TUNEL staining was undertaken by Dr Razao Issa (Postdoctoral fellow) and slides were counted by myself. (Data are expressed as mean +/- SEM percentage TUNEL positive cells; **$p<0.005$; n=2)

3.2.5 TIMP-1 inhibits apoptosis induced by Nerve Growth factor

We have previously demonstrated that HSC express low affinity nerve growth factor receptor (p75) and undergo apoptosis in response to Nerve growth factor (NGF) stimulation via the extrinsic apoptotic pathway (Trim et al. 2000). To determine if TIMP-1 reduced NGF induced apoptosis NGF activated HSC were exposed to NGF (100ng/ml) in conditions of absolute serum deprivation with and without TIMP-1 (142.5ng/ml). As expected, NGF induced significantly more apoptosis in HSC than cells treated with BSA carrier alone. TIMP-1 significantly reduced the apoptosis induced by Nerve growth factor (figure 3.7).

FIGURE 3.7. EFFECT OF TIMP-1 ON NGF INDUCED APOPTOSIS IN ACTIVATED HSCS.

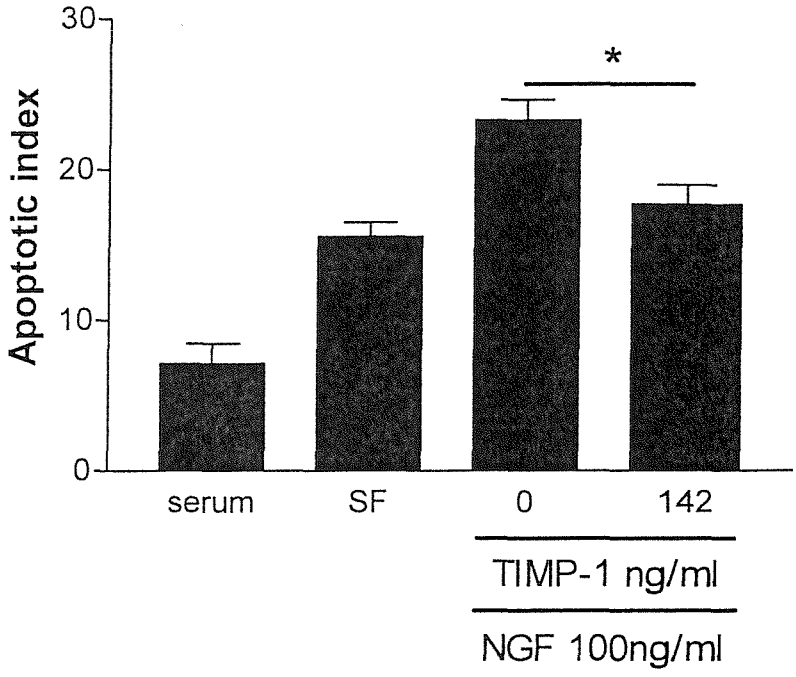


Figure 3.7. To determine whether TIMP-1 could reduce apoptosis induced by NGF, activated rat HSC were incubated in serum and serum free conditions. Apoptosis induced by exposure to NGF (100ng/ml) in serum free conditions, was significantly inhibited by TIMP-1 (5nM or 142.5ng/ml). Apoptosis was quantified by the acridine orange technique. (Data are expressed as mean +/- SEM apoptotic index; *$p<0.05$ for NGF treated alone versus NGF with TIMP-1 treatment by Student's t-test; n=3).

3.2.6 TIMP-1 and TIMP-2 inhibit apoptosis induced by gliotoxin

The fungal metabolite gliotoxin has previously been shown to induce apoptosis of HSC via activation of the intrinsic apoptotic pathway via the mitochondria (Wright et al. 2001). Human cultured HSC exposed to TIMP-1 or TIMP-2 had significantly reduced apoptosis after exposure to gliotoxin (1.5μM) for four hours (Figure 3.8).

FIGURE 3.8. EFFECT OF TIMP-1 AND TIMP-2 ON GLIOTOXIN INDUCED APOPTOSIS OF CULTURED HUMAN HSC

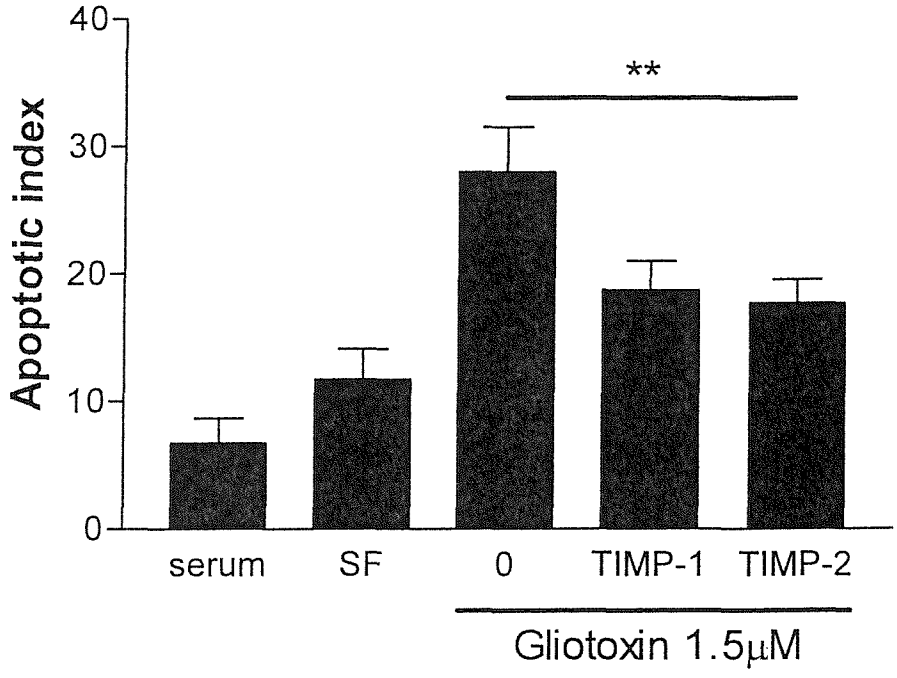


Figure 3.8. To determine whether TIMP-1 or TIMP-2 could reduce apoptosis induced by gliotoxin, activated human HSC were incubated in serum and serum free conditions. Apoptosis induced by exposure to gliotoxin (1.5μM) for four hours in serum free conditions, was significantly inhibited by TIMP-1 (20nM or 560ng/ml) or TIMP-2 (20nM or 420ng/.ml). Apoptosis was quantified by the acridine orange technique. (Data are expressed as mean +/- SEM apoptotic index; **$p<0.005$ for gliotoxin treated alone versus gliotoxin with TIMP-1 or TIMP-2 treatment by Student's t-test; n=3).

3.2.6 TIMP-1 and TIMP-2 are autocrine survival factors for HSCs.

It is known that TIMP-1 and TIMP-2 are major synthetic products of activated HSC (Arthur et al. 1993). Therefore, TIMP-1 and TIMP-2 are potentially autocrine survival factors for HSC. To determine the effect of neutralising HSC derived TIMP-1 and TIMP-2, HSC were incubated with azide free polyclonal neutralising antibodies to TIMP-1 and TIMP-2 for 18 hours in 5% bovine serum albumin. This demonstrated significantly increased apoptosis of HSC treated with TIMP neutralising antibodies compared to a non-immune IgG control suggesting that TIMP-1 and TIMP-2 act as survival factors in an autocrine manner for activated HSCs (Figure 3.9).

FIGURE 3.9. TIMP-1 AND TIMP-2 NEUTRALISING ANTIBODIES INCREASE APOPTOSIS OF ACTIVATED HSCS.

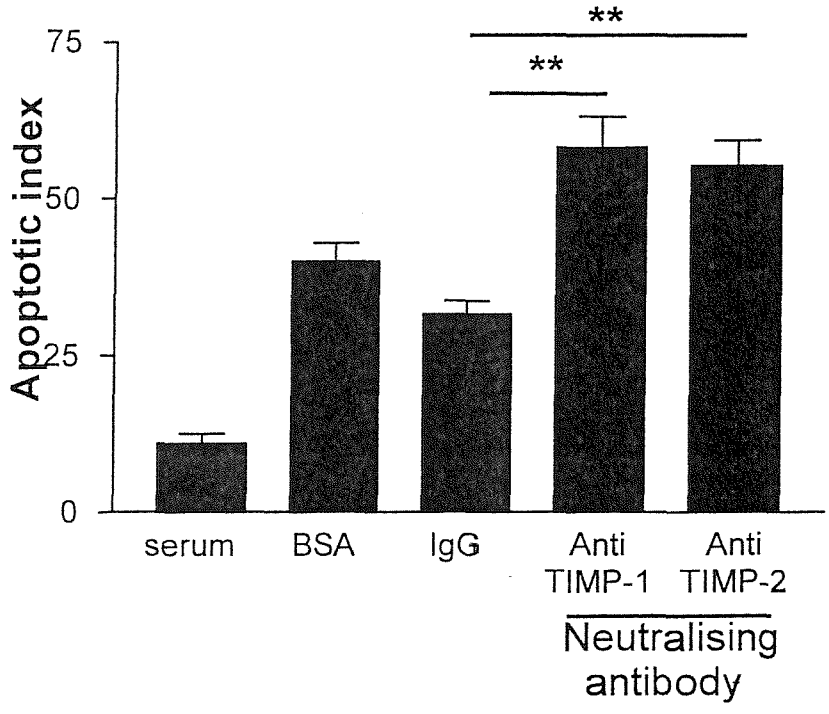


Fig 3.9. Activated rat hepatic stellate cells were incubated in serum free conditions with 5% BSA for 18 hours in the presence and absence of neutralising antibodies to TIMP-1 and TIMP-2 compared to a non immune IgG control antibodies as described in the methods. Neutralising TIMP-1 and TIMP-2 significantly increased apoptosis of activated rat HSC compared to exposure to the non immune IgG control. All antibodies were in azide free buffer. Apoptosis was quantified by the acridine orange technique. (Data are expressed as mean +/- SEM apoptotic index after 18 hours; **$p<0.005$ by Student's t-test for HSC treated with neutralising antibodies for TIMP-1 and TIMP-2 relative to non immune IgG control; n=3)

3.2.7 TIMP-1 and TIMP-2 have no effect on HSC proliferation.

Because previous studies in other cell types have demonstrated a potential pro proliferative effect for TIMP-1 and TIMP-2 this was analysed in activated rat HSC cultured on plastic. Neither TIMP-1 nor TIMP-2 at concentrations of 1-100ng/ml had any effect on proliferation of rat HSC over a 24 hr incubation period compared to bovine serum albumin carrier used as a negative control (Figure 3.10). Serum containing media (16%) was used as a positive control.

FIGURE 3.10 TIMP-1 AND TIMP-2 HAVE NO EFFECT ON PROLIFERATION OF ACTIVATED RAT HSC CULTURED ON PLASTIC

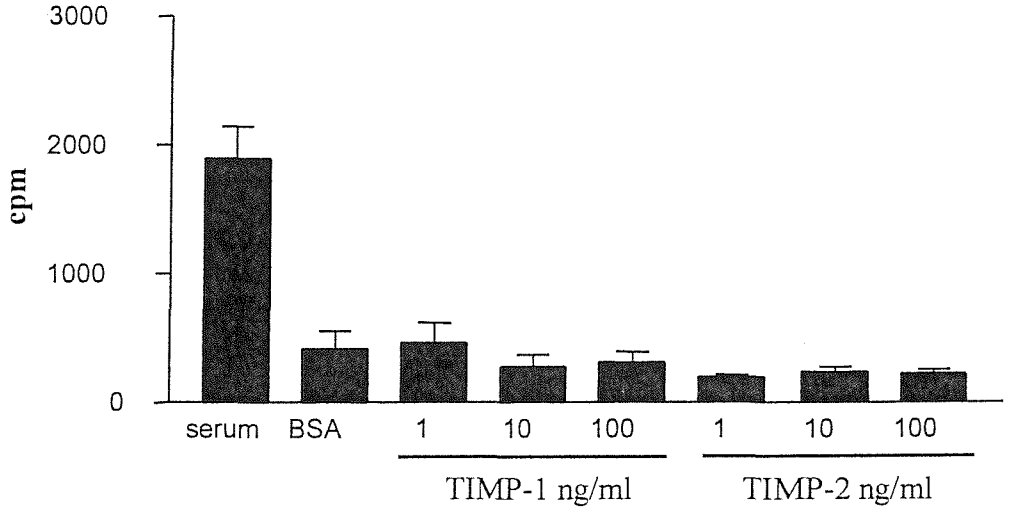


Figure 3.10 Rat activated HSC culture on plastic were used to determine if TIMP-1 or TIMP-2 could change the rate of cellular proliferation. Proliferation was quantified by the tritiated thymidine technique as described in the methods. Neither TIMP significantly increased proliferation at a concentration range of 1-100ng/ml. There was a trend for TIMP-2 to reduce proliferation but it did not reach statistical significance (Data are expressed as mean +/- SEM counts per minute (cpm), each condition was undertaken in triplicate, n=5, meaning five separate experiments each using HSC extracted from a different animal).

3.2.8 Summary of in vitro experiments.

1. The in vitro experiments showed a consistent, reproducible, dose dependent anti apoptotic effect for TIMP-1 and to a lesser extent TIMP-2 for activated rat and human hepatic stellate cells.
2. The anti apoptotic effects observed for TIMP-1 and TIMP-2 by acridine orange staining and counting correlated very well with parallel experimental data obtained using Caspase-3 assay and quantification of DNA fragmentation using the TUNEL technique.
3. TIMP-1 and TIMP-2 are autocrine survival factors for activated HSC.
4. Neither TIMP-1 nor TIMP-2 effects proliferation of activated rat HSC cultured on plastic.

3.3 Discussion

3.3.1 These studies have provided three lines of evidence that together have demonstrated TIMP-1 promotes survival of activated hepatic stellate cells by inhibiting their apoptosis. Firstly, contrasting the 6 and 12 week carbon tetrachloride models of liver fibrosis/cirrhosis demonstrated that correlation exists between falling TIMP-1 expression, loss of activated HSC, resolution of liver fibrosis and transient increases in collagenolytic activity in liver homogenates. Secondly, apoptosis of HSC quantified by TUNEL staining was enhanced in the TIMP-1 knockout mice compared to wild type mice in another model of liver fibrosis. Thirdly, the in vitro studies demonstrate that TIMP-1 and TIMP-2 directly inhibit apoptosis of activated HSC and that they are autocrine survival factors. Previous studies have shown during recovery from liver fibrosis in the rat carbon tetrachloride (Iredale et al. 1998) and bile duct ligation model of fibrosis (Issa et al. 2001) there is a diminution of HSC number mediated by apoptosis. At the same time there is a reduced expression of TIMP-1. These studies and the data described here have addressed a crucial question to our understanding of liver fibrosis: What determines whether a fibrotic liver injury recovers or fails to recover? The starting point for the studies described in this chapter was the observation that in recovery there is a net reduction in activated HSCs and fibrotic matrix, while in progressive fibrosis the activated HSCs and neo-matrix remain. Identification of factors promoting the survival of activated HSC is therefore essential to understanding the pathogenesis of fibrosis. TIMP-1 and TIMP-2 are important potential candidates mediating HSC survival.

3.3.2 The studies I have described represent a series of experiments using the established and robust model of activated hepatic stellate cells in tissue culture. I have analysed the influence of TIMP-1 and TIMP-2 on HSC apoptosis induced by a variety of stimuli. The results in tissue culture indicate that TIMP-1 and to a lesser extent TIMP-2 have a direct, consistent, significant and concentration dependent anti-apoptotic effect on both human and rat HSC. I have shown, using a series of complementary quantitative techniques, TIMP-1 reduces apoptosis induced by serum deprivation, cycloheximide or gliotoxin exposure and nerve growth factor stimulation and that this effect is shared by both rat and human HSC, suggesting that it is a biologically important phenomenon. An interesting observation from the different methods used to quantify apoptosis is that while acridine orange staining of cells and counting was a highly sensitive technique, assessment of caspase-3 activity of TUNEL staining and cell counting did not always show similar dose effects and to some

extent a discrepancy was observed between the results obtained by different methods. For example, acridine data from the dose response of TIMP-1 induced apoptosis in rat HSC (Figure 3.3a) showed 200ng/ml TIMP-1 reduced apoptosis induced by exposure to cycloheximide by 60%. Parallel experiments assessing apoptosis by caspase-3 activity (Figure 3.5) or TUNEL staining (Figure 3.6) measured decreases of 50% and 52% respectively. It is not surprising that given the methodological differences of the three techniques that there was some observed variation in precision and sensitivity between the three different techniques. What is important is that the overall trend of the results were the same and were reproducible.

3.3.3 Neither TIMP-1 nor TIMP-2 had any pro proliferation effect on activated HSCs. From a biological view, it would seem undesirable for a protein to both inhibit apoptosis and promote proliferation in the same cell type as expression of such a protein would be potentially carcinogenic. Experiments to assess proliferation in rat activated HSC were designed so that both increases or decreases in proliferation could be detected. Clearly TIMP-1 did not appear to effect proliferation in low serum conditions, however, the dose response for TIMP-2 had an inhibitory trend but this was not significant (see figure 3.10).

3.3.4 Together, the data I have described in this chapter provide strong evidence that TIMP-1 and TIMP-2 are mechanistically important in promoting fibrosis: Firstly by directly inhibiting MMPs thus promoting matrix accumulation, and secondly, by inhibiting the apoptosis of activated hepatic stellate cells.

Chapter 4

4. Mechanistic studies of the anti apoptotic effect of TIMP-1.

4.1 Introduction: The overall approach to the mechanistic study.

In the last chapter I described in some detail the basic observation that TIMP-1 and TIMP-2 inhibit apoptosis of activated HSC. The strategy to unravel the mechanism mediating the anti apoptotic effect was as follows. Firstly, the effect of a mutated TIMP-1 was studied. The mutant TIMP-1 used was the T2G mutant N-TIMP-1 and its wild type control. These were supplied by Professor Hideaki Nagase and were manufactured as described in the methods and materials by Dr Q. Meng under the supervision of Dr Keith Brew of the University of Miami Medical School. The second part of the approach was to make use of the wide variety of commercially available MMP inhibitors to screen for selective MMP inhibitors that could also inhibit apoptosis of cultured HSC. Following these studies, recombinant active MMPs were used and their effect on human HSC apoptosis examined. The third line of investigation was to use a gene array of human apoptosis related genes and examine the changes in extracted mRNA from human HSC that where either exposed to excess exogenous wild type N-TIMP-1 or the T2G mutant N-TIMP-1. Candidate genes showing changes were further studied at the protein level by ELISA or western blot. The fourth line of mechanistic investigation was to examine the effect of TIMPs on active caspase-3 activity and to examine the fate of TIMP-1 during and after apoptosis of human HSC.

4.2 The anti apoptotic effect of TIMP-1 for HSC is mediated via MMP inhibition.

Previous studies have suggested that the observed anti apoptotic effect of TIMP-1 in other cell systems was independent of MMP inhibition (Guedez et al. 1998). The experiments described in the previous chapter show an effective anti apoptotic activity for TIMP-1 and TIMP-2 suggesting that the effect might be mediated via a common mechanism, such as MMP inhibition. Further experiments using a mutated non functional T2G N-TIMP-1 were undertaken (Meng et al. 1999). The inhibitory effect of wild type and T2G mutant N-TIMP-1 are shown in Table 4.1. The T2G mutant N-TIMP-1 had no inhibitory effect on rat or human hepatic stellate cell apoptosis induced by cycloheximide, while the wild type N-TIMP-1 protein at identical concentration (142.5ng/ml or 5nM) significantly inhibited apoptosis (Figure 4.1a & 4.1b) determined by in situ counting after acridine orange staining. Moreover, whilst the wild type TIMP-1 reduced Caspase-3 activity in HSC treated with cycloheximide,

no effect was observed with the T2G non functional mutant (Figure 4.2). The observations made by the acridine orange and Caspase-3 assay correlated well with each other and suggest that the inhibition of HSC apoptosis by TIMP-1 was MMP dependent.

FIGURE 4.1A THE T2G MUTANT TIMP-1 DOES NOT INHIBIT APOPTOSIS OF RAT ACTIVATED HSC INDUCED INTO APOPTOSIS BY CYCLOHEXIMIDE

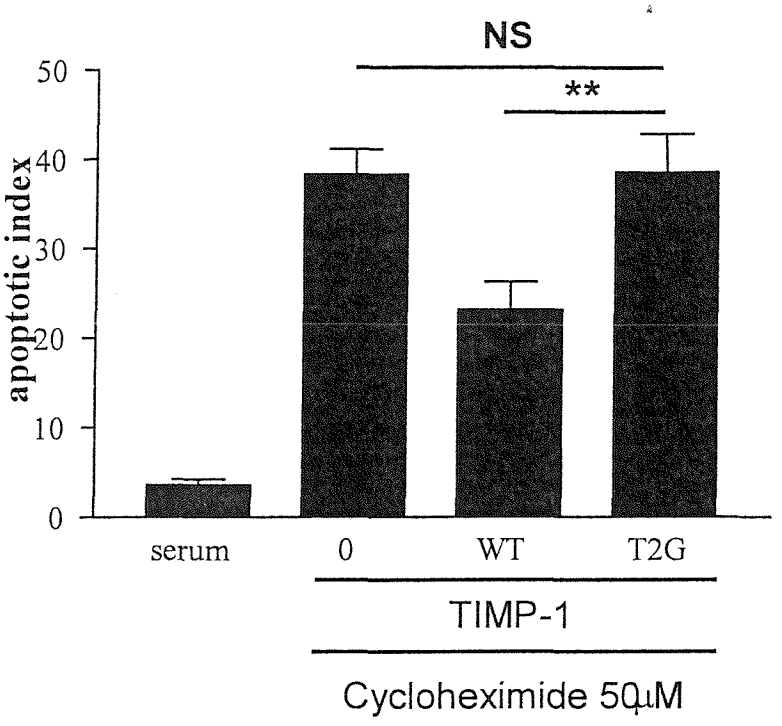


Figure 4.1a. Acridine orange staining and counting of rat HSC exposure to cycloheximide with and without wild type and T2G mutant TIMP-1, demonstrated that while the wild type TIMP-1 significantly inhibited apoptosis, the T2G mutant N-TIMP-1 (with no MMP inhibitory activity) had no effect on apoptosis at equal concentration (142.5ng/ml or 5nM). (Data are expressed as mean +/- SEM apoptotic index; *$p<0.05$ by Student's t-test. NS= not significant by Student's t-test, n=3)

FIGURE 4.1B THE T2G MUTANT TIMP-1 DOES NOT INHIBIT APOPTOSIS OF HUMAN ACTIVATED HSC INDUCED INTO APOPTOSIS BY CYCLOHEXIMIDE

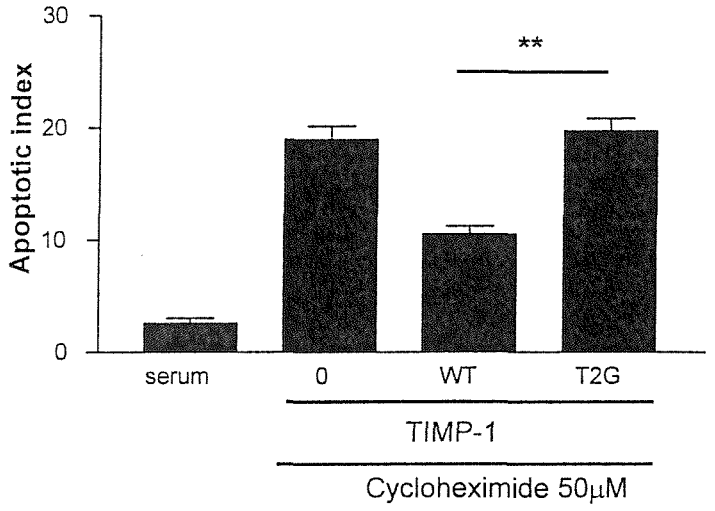


Figure 4.1b Acridine orange staining and counting of human HSC exposure to cycloheximide with and without wild type and T2G mutant TIMP-1, demonstrated that while the wild type TIMP-1 significantly inhibited apoptosis, the T2G mutant N-TIMP-1 (with no MMP inhibitory activity) had no effect on apoptosis at equal concentration (142.5ng/ml). (Data are expressed as mean +/- SEM apoptotic index; **$p<0.005$ by Student's t-test. n=1)

FIGURE 4.2 CASPASE-3 ACTIVITY OF RAT HSC INDUCED INTO APOPTOSIS BY CYCLOHEXIMIDE IS REDUCED BY WILD TYPE TIMP-1 BUT NOT BY THE T2G MUTANT TIMP-1

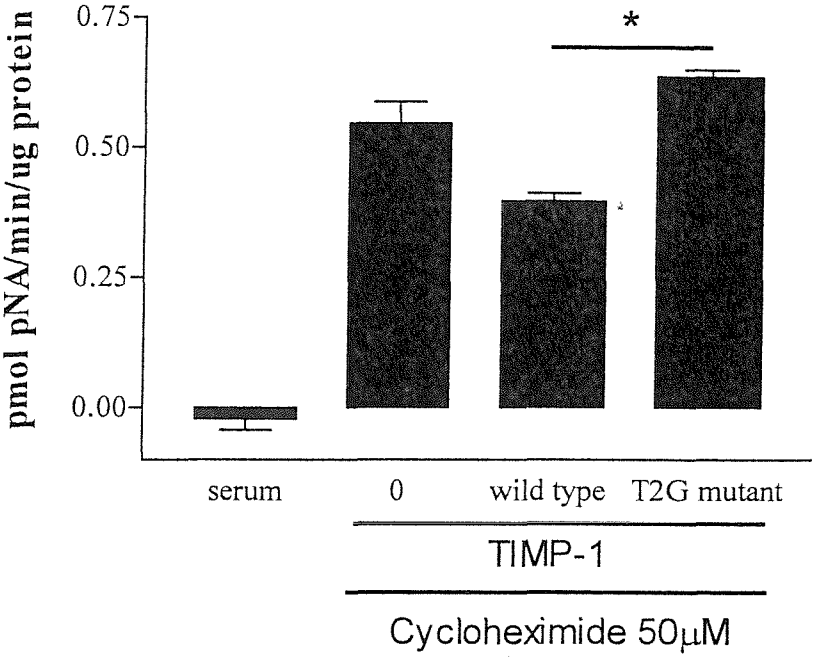


Fig 4.2. Graph demonstrating that the Caspase-3 activity of cell extracts from rat HSC treated with cycloheximide in the presence of wild type (active) TIMP-1 and the T2G mutant (which has no MMP inhibitory activity). The wild type TIMP-1 significantly reduced Caspase-3 activity relative to the T2G mutant. (Data presented as mean+/-SEM; $p<0.05$; n=3)

4.3 The choice of MMP inhibitors for further study of MMP inhibition on HSC apoptosis.

To determine which MMP or MMPs were involved in HSC apoptosis, a panel of selective and semi-selective MMP inhibitors was obtained (Calbiochem, UK). Previous literature described in the introduction has shown that HSC express MMP-1, MMP-2, MMP-3, MMP-9 and MMP-14 or MT1-MMP. Experiments were undertaken with HSC induced into apoptosis by cycloheximide with and without the panel of selective MMP inhibitors. Table 4.1 describes the characteristics of the inhibitors chosen.

Human cells express MMP-1 but not MMP-13. For this reason human HSC were used in experiments to allow use of the MMP-9/13 Inhibitor II as a semi selective inhibitor of MMP-9 as the K_I for MMP-1, -3, and 7 are at least 10 fold higher in concentration. For inhibition of MMP-1, GM1489 was used because its K_I for MMP-1 is 0.2nM while that for MMP-2, -3, -8 and –9 are at least 1000 fold higher in concentration.

TABLE 4.1. CHARACTERISTICS OF MMP INHIBITORS USED:

MMP inhibitor	MMPs inhibited:	K_I	Concentration used:
Wild type N-TIMP-1	MMP-1 MMP-2 MMP-3	3.0nM 1.1nM 1.9nM	5nM
Mutant T2G N-TIMP-1	MMP-1 MMP-2 MMP-3	18μM 103μM 1380nM	5nM
GM1489	MMP-1 MMP-2 MMP-3 MMP-8 MMP-9	0.2nM 500nM 20μM 100nM 100nM	0.2nM
MMP-2 Inhibitor I	MMP-2	1.7μM	1.7μM
MMP-3 Inhibitor II	MMP-3	130nM	130nM
MMP-9/13 Inhibitor II	MMP-1 MMP-3 MMP-7 MMP-9 MMP-13	24nM 18nM 230nM 1.9nM 1.3nM	1.9nM
MMP Inhibitor I	MMP-1 MMP-3 MMP-8 MMP-9	1.0μM 150μM 1.0μM 30μM	1.0μM 1.0μM 30μM

4.4 A comparison of TIMP-1, TIMP-2 and MMP inhibitor I.

Parallel experiments were undertaken with the synthetic MMP inhibitor (MMPI-1, Calbiochem, UK). The concentration of inhibitor used was calculated to provide a comparable level of MMP inhibition to 142.5ng/ml TIMP-1 on the basis of the published K_I for the inhibitor and the recombinant TIMP-1. The synthetic matrix metalloproteinase inhibitor MMPI-1 also demonstrated a dose dependent protective effect at a concentration of 1-30μM. This suggested that the anti-apoptotic effect in HSC could be brought about by matrix metalloproteinase inhibition alone (Figure 4.3).

FIGURE 4.3 COMPARISON OF COMMERCIAL MMP INHIBITOR MMPI-1, TIMP-1 AND TIMP-2 ON RAT HSC APOPTOSIS INDUCED BY CYCLOHEXIMIDE EXPOSURE

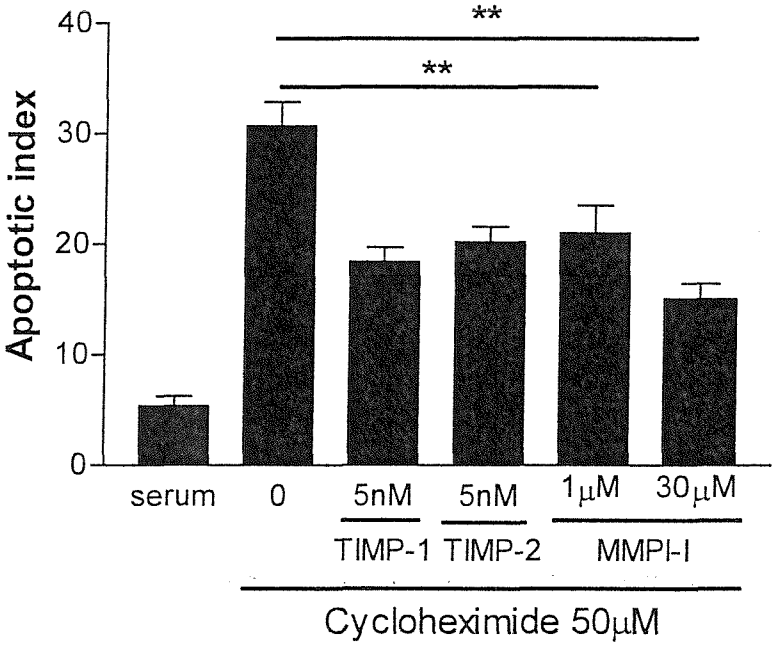


Figure 4.3. Studies comparing the anti apoptotic effect of TIMP-1 (142.5ng/ml, 5nM) and TIMP-2 (105ng/ml, 5nM) and the synthetic MMP inhibitor MMPI-1 were undertaken as described in the methods. As previously described TIMP-1 and TIMP-2 inhibited apoptosis induced by cycloheximide exposure. At a concentration of 1 to 30 μM, MMPI-1 significantly inhibited HSC apoptosis induced by cycloheximide determined by acridine orange staining and cell counting (data are expressed as mean +/- SEM apoptotic index; **p<0.005 Student's t-test, n=3)

4.5 Synthetic MMP inhibitors that reduced apoptosis.

Using a panel of semi selective and selective MMP inhibitors at comparable inhibitory concentrations as described in Table 4.1, human HSC were responsive to significant apoptosis

inhibition by the MMP inhibitors (Figure 4.4a). It is not possible to conclude from this experiment which MMP might play a role in human HSC apoptosis because the panel of inhibitors is at best only likely to be semi selective. However, this data does emphasize that inhibition of MMPs is one mechanism to reduce apoptosis. Similar data were obtained in parallel experiments where apoptosis was quantified by Caspase-3 activity (Figure 4.4b).

FIGURE 4.4A COMPARISON OF THE EFFECT OF SELECTIVE INHIBITION OF MMP ON CYCLOHEXIMIDE INDUCED APOPTOSIS

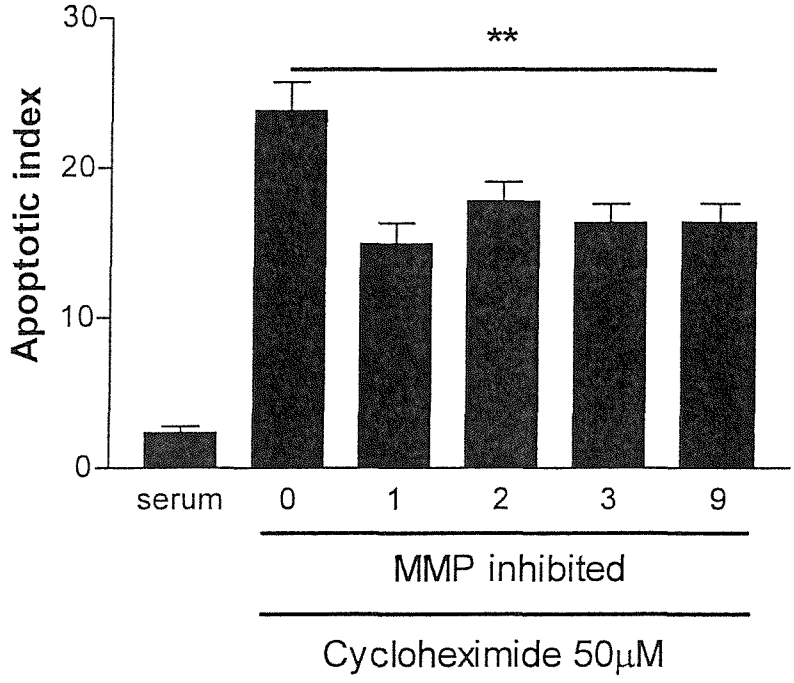


Figure 4.4a. Comparison of the effects of a panel of MMP inhibitors at concentrations chosen to selectively inhibit MMPs indicated that all had some significant effect of HSC apoptosis induced by cycloheximide. Apoptosis was determined by acridine orange staining and cell counting (data are expressed as mean +/- SEM apoptotic index; **$p<0.005$ by Student's t-test for cycloheximide versus all MMP inhibitors, n=3).

FIGURE 4.4B CASPASE-3 ACTIVITY ASSAYS OF SELECTIVE MMP INHIBITORS

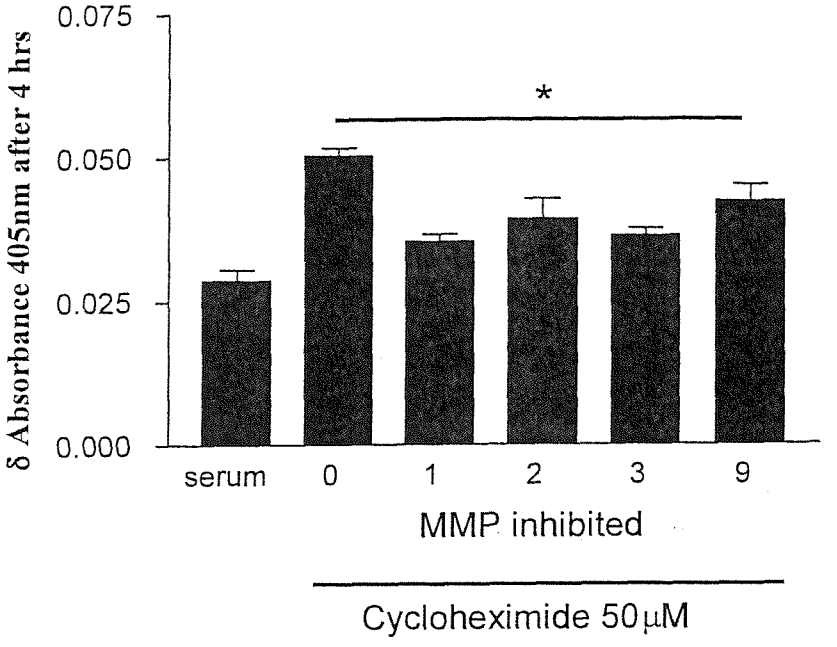


Figure 4.4b. To verify acridine orange counting observations parallel experiments were undertaken and apoptosis measured by caspase-3 activity assay. (Data presented are representative of three separate experiments, *$p<0.05$, by Student's t test, n=3).

4.6 Effect of active MMPs on HSC apoptosis.

To complement the MMP inhibitor studies active MMPs were obtained (Calbiochem,UK). Active MMP-2, the catalytic domain of MMP-3, and active MMP-9 were used in experiments on human cultured HSC. The activity of the MMPs was verified by gelatin zymography (Figure 4.5a).

FIGURE 4.5A GELATIN ZYMOGRAPHY OF SUPPLIED ACTIVE MMP-2 AND ACTIVE MMP-9

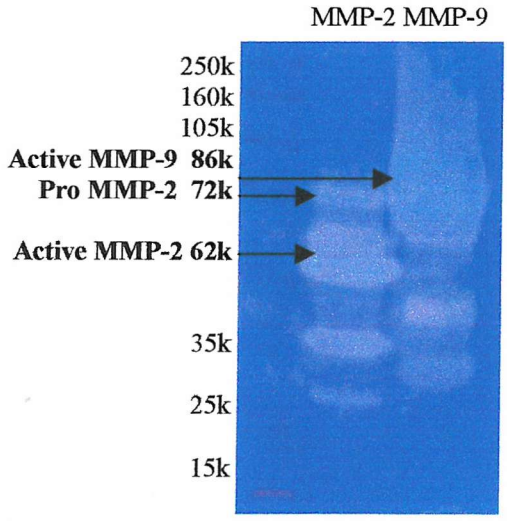


Figure 4.5a. This gelatin zymogram shows that the two MMPs used in further experiments was in its active form. A small amount of proMMP-2 (72k band) is present but the majority of the protein is in its active 62k form in addition to smaller autocatalytic bands. A similar appearance is found with the supplied MMP-9. The proMMP-9 (92k band) is not clearly visible but there is an obvious large band at 86k which represents the active form of MMP-9. There are in addition smaller autocatalytic bands present.

4.6.1 Cultured human HSC were exposed to the active MMPs in serum free conditions with and without cycloheximide to facilitate apoptosis. Apoptosis was initially quantified by acridine orange counting. Equimolar quantities of each MMP were compared. Active MMP-2 was the most potent at promoting apoptosis of human HSC followed by MMP-9 and MMP-3 (Figure 4.5b, c and d). Approximately 40% of the cells had apoptotic morphology after 4 hours exposure to 20nM active MMP-2 with cycloheximide. MMP-9 and MMP-3 (22k catalytic domain) showed a similar pattern but neither could match the potency of active

MMP-2 at promoting apoptosis. For this reason further studies (see chapter 5) focused on active MMP-2 as the major MMP involved in apoptosis.

FIGURE 4.5B EFFECT OF ACTIVE MMP-2 ON HUMAN HSC APOPTOSIS

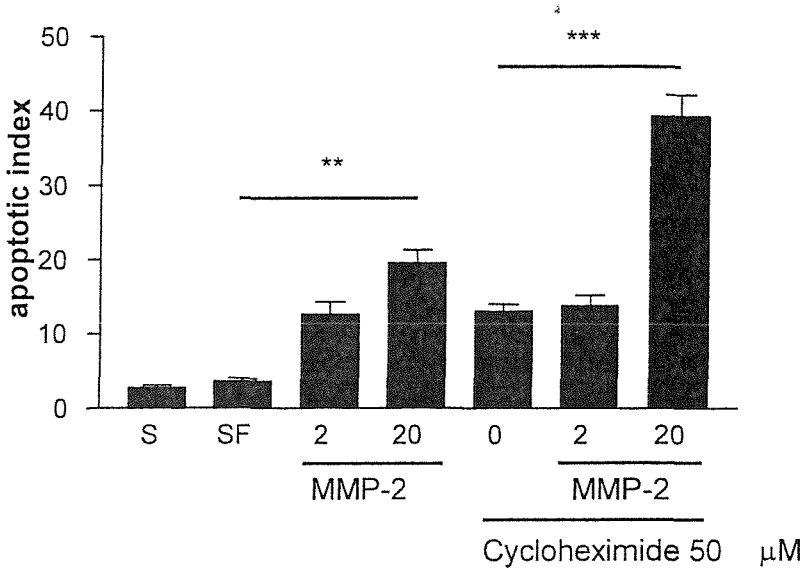
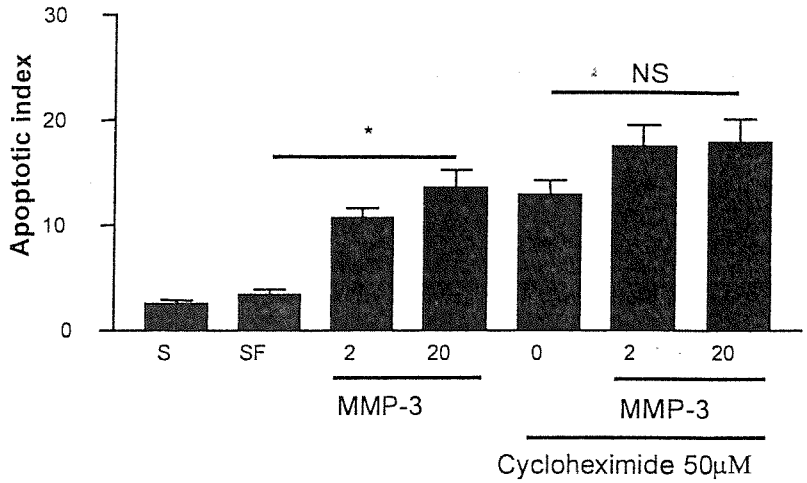


Figure 4.5b. Acridine orange staining was used to quantify apoptosis of human HSC after exposure to 2nM or 20nM of active recombinant human MMP-2 with and without cycloheximide for 4 hours. Data shown are mean and SEM apoptotic index of three separate experiments. **$p<0.005$, ***$p<0.0005$, by Student's t test.

FIGURE 4.5C & 4.5D. EFFECTS OF ACTIVE MMP-3 AND MMP-9 ON HUMAN HSC APOPTOSIS

C.



D.

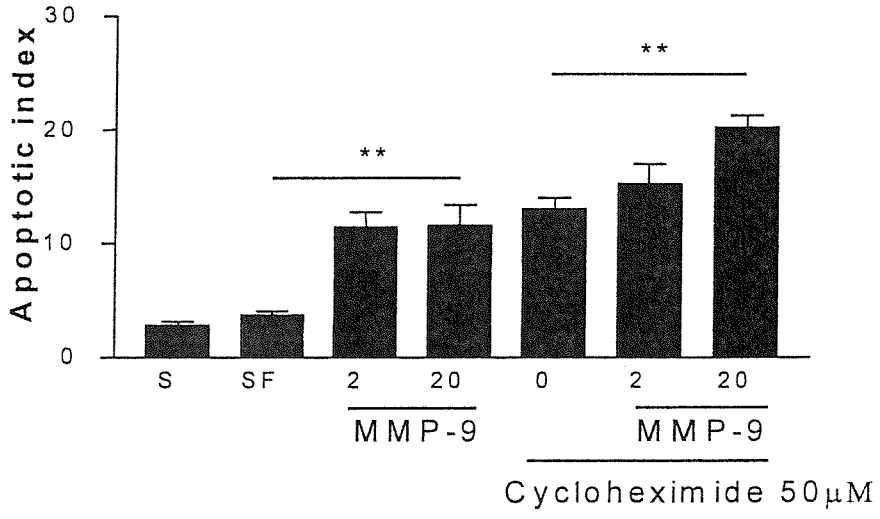


Figure 4.5c & d. Acridine orange staining was used to quantify apoptosis of human HSC after exposure to a panel of active MMPs for 4 hours. Data shown are mean and SEM apoptotic index of three separate experiments. *p<0.05, **p<0.005 by Student's t test.

4.7 Effects of TIMP-1 on apoptosis related mRNA

Further studies were undertaken to determine if treatment of activated HSC with exogenous TIMP-1 effects mRNA for apoptosis related genes. RNA extracted was of good purity and quality judged by agarose gel electrophoresis integrity gel (Figure 4.6).

FIGURE 4.6A RNA INTEGRITY GEL EXAMPLE

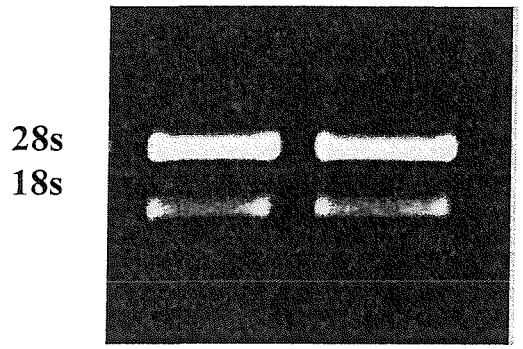


Figure 4.11 An example of an RNA integrity gel demonstrating that RNA extracted for activated human HSC was of excellent purity and quality. (Lanes were loaded with 1μg of total cellular RNA).

4.7.1 To determine which genes might be of importance in the mechanism by which TIMP-1 inhibited apoptosis, human HSC were exposed in serum free conditions for 18 hours to 5nM of either wild type TIMP-1 or the non functional T2G N-TIMP-1 protein. Extracted mRNA was used in a gene array with 96 candidate genes as described in the methods. Visible spots were quantified from 43 out of the 96 target genes. All 12 house keeping genes gave strong signals and the 3 bacterial plasmid and 3 blank negative control spots had no signal evident (Figure 4.6b).

4.7.2 There are a number of limitations to this type of gene analysis. Firstly, there are approximately 40 shades of grey on a standard X-ray film and so the maximum fold change that could be quantified by this technique would be 40 fold. For this reason the results of the image analysis program have been expressed as colour changes rather than numerically. In figure 4.6c the strength of the signal has been grouped into four: BLACK (no signal), YELLOW (weak signal), GREEN (medium signal) and RED (strong signal). For gene expression changes weak to moderate signals are likely to be more quantitative than the strongest signals. In Figure 4.6d the fold change in spot intensity has been expressed as a

colour: BLACK (no signal), RED: no change, GREEN: 2-10 fold increase, BLUE: greater than 10 fold increase.

4.7.3 The genes that were detectable with the array included 11 genes known to inhibit apoptosis and 32 genes known to promote apoptosis (Figure 4.6c). The three strongest apoptosis inhibitory signals were observed for MCL-1 (spot 7.4 in Figure 4.6b)(myeloid cell leukaemia sequence 1, Bcl-2 related), TRAIL receptor 4 (spot 9.3 in Figure 4.6b) and TNFreceptor associated factor 1 (TRAF 1, spot 12.2 in Figure 4.6b). The four strongest apoptosis promoting signals were observed for BAK (Bcl2-antagonist/killer 1, spot 1.5 in Figure 4.6b), BLK (B lymphoid tyrosine kinase, spot 3.4 in Figure 4.6b), caspase-14 (spot 4.2 in Figure 4.6b) and TNF receptor 2 (spot 9.7 in Figure 4.6b).

4.7.4 The semi quantitative analysis of the gene spot intensity relative to GAPDH showed that, of the 11 genes with apoptosis inhibitory effects, expression of 3 genes was not changed by TIMP-1, while the remaining 8 genes were increased by exposure to TIMP-1 compared to the T2G N-TIMP-1. A similar study of the 33 detectable apoptosis promoting genes revealed that exposure to TIMP-1 had no effect on expression of 19 genes but increased expression in 14 genes.

4.7.5 The major limitation with this gene expression study is that it became clear that many genes were changed by exposure to TIMP-1 compared to the T2G N-TIMP-1. It is not possible to conclude which gene or group of genes has a dominant role in the overall effect of inhibition of human HSC apoptosis by TIMP-1. Thus gene array data can at best be considered a screen of which genes might be important. An interesting observation was that nearly all genes that changed were increases rather than decreases. This suggests the MMP inhibition by TIMP-1may yield a cellular phenotype that both is inhibited from apoptosis but with enhanced latent apoptosis mechanisms so that should the appropriate apoptotic stimulus arrive (or survival signal be withdrawn) then apoptosis would be facilitated.

FIGURE 4.6B THE HUMAN APOPTOSIS GENE ARRAY EXAMPLE

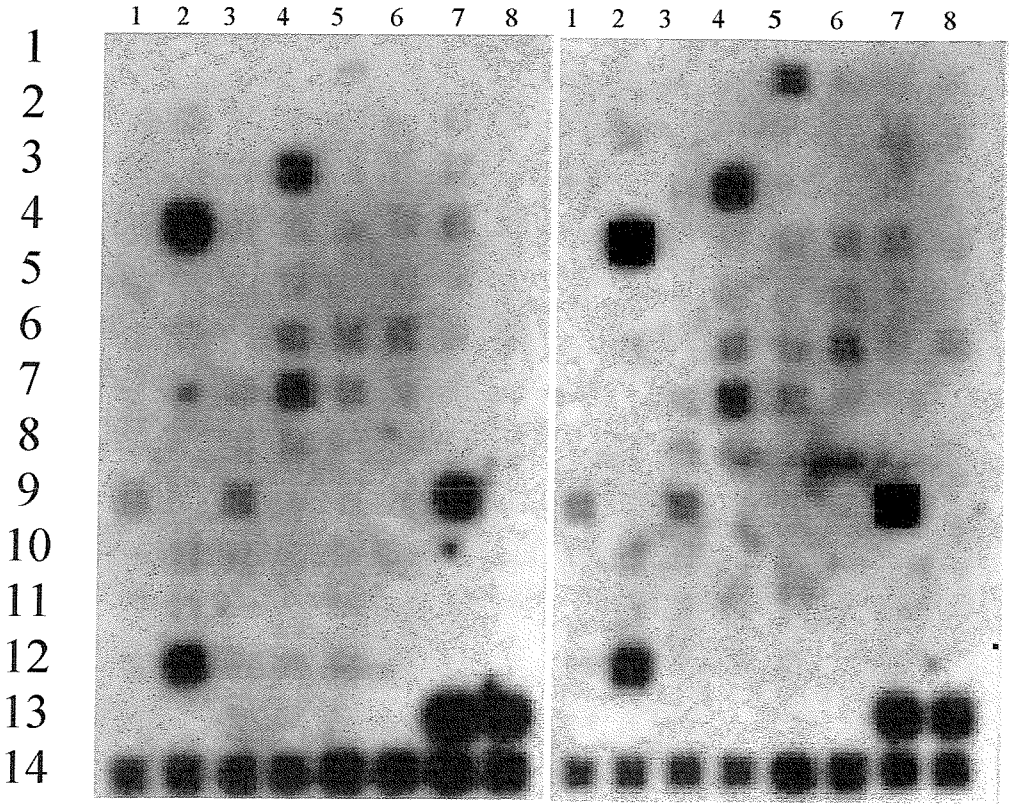


FIGURE 4.6C GENE SIGNAL STRENGTHS FROM TIMP-1 ARRAY SPOTS:

	1	2	3	4	5	6	7	8
1	APAF1	ASC	ATM	BAD	BAK1 +	BAX +	BCL10	BCL2 -
2	BCL2A1	BCL2L1 -	BCL2L11	BCL2L2	BIK	BIRC1	BIRC2 -	BIRC3 -
3	BIRC4 -	BIRC5	BIRC6 -	BLK +	BNIP3	BOK +	CASP1 +	CASP10
4	CASP13	CASP14 +	CASP2 +	CASP3 +	CASP4 +	CASP5 +	CASP6 +	CASP7
5	CASP8 +	CASP8AP 2	CASP9	CFLAR +	CHEK1	CIDEA +	CIDEB +	CRADD
6	DAPK2	DFFA +	DFFB	FADD +	GADD45 A+	HRK +	HUS1	BAR -
7	LTA	LTB	LTBR +	MCL1 -	MDM2 +	MYD88 +	NOD1	NOL3
8	P63	RAD53	RIPK1 +	RIPK2 +	RPA3	TANK	TNF	TRAIL R
9	TRAIL R2+	TRAIL R3	TRAIL R4-	DDR3	TR2	TNFR SF1A	TNFR2 +	TNFR SF4
10	TNFR SF5	FAS +	TNFR SF7-	TNFR SF8	TNFR SF9	TNF SF10	TNF SF11	TNF SF12
11	TNF SF13	TNF SF14+	TNF SF4	TNF SF5	FAS+ LIGAND	TNF SF7	TNF SF8	TNF SF9
12	TP53	TRAF1 -	TRAF2 -	TRAF3 +	TRAF4 +	TRAF5	TRAF6	TRIP
13	PUC18	PUC18	PUC18	BLANK	BLANK	BLANK	GAPDH	GAPDH
14	PPIA	PPIA	PPIA	PPIA	RPL13A	RPL13A	ACTB	ACTB

Figure 4.6c. Semi quantitative analysis of mRNA from human HSC exposed to wild type TIMP-1 in serum free conditions for 18 hours. For simplicity, signal strength is expressed as a colour: BLACK: no signal, YELLOW: weak signal, GREEN: medium signal, RED: strong signal. Detectable signal was observed with 12 genes that inhibit apoptosis (-) and 32 genes known to promote apoptosis (+).

FIGURE 4.6D SEMI QUANTITATIVE EXPRESSION OF GENE ARRAY CHANGES:

	1	2	3	4	5	6	7	8
1	APAF1	ASC	ATM	BAD	**BAK1** +	**BAX** +	BCL10	**BCL2** -
2	BCL2A1	**BCL2L1** -	BCL2L11	BCL2L2	BIK	BIRC1	**BIRC2** -	**BIRC3** -
3	**BIRC4** -	BIRC5	**BIRC6** -	**BLK** +	BNIP3	**BOK** +	**CASP1** +	CASP10
4	CASP13	**CASP14** +	**CASP2** +	**CASP3** +	**CASP4** +	**CASP5** +	**CASP6** +	CASP7
5	**CASP8** +	CASP8AP 2	CASP9	**CFLAR** +	CHEK1	**CIDEA** +	**CIDEB** +	CRADD
6	DAPK2	**DFFA** +	DFFB	**FADD** +	**GADD45 A+**	**HRK** +	**HUS1** +	BAR -
7	LTA	LTB	**LTBR** +	**MCL1** -	**MDM2** +	**MYD88** +	NOD1	NOL3
8	P63	RAD53	**RIPK1** +	**RIPK2** +	RPA3	TANK	TNF	TRAIL R
9	**TRAIL R2+**	TRAIL R3	**TRAIL R4-**	DDR3	TR2	TNFR SF1A	**TNFR2** +	TNFR SF4
10	TNFR SF5	**FAS** +	**TNFR SF7+**	TNFR SF8	TNFR SF9	TNF SF10	TNF SF11	TNF SF12
11	TNF SF13	**TNF SF14+**	TNF SF4	TNF SF5	**FAS+ LIGAND**	TNF SF7	TNF SF8	TNF SF9
12	TP53	**TRAF1** -	TRAF2 -	TRAF3 +	TRAF4 +	TRAF5	TRAF6	TRIP
13	PUC18	PUC18	PUC18	BLANK	BLANK	BLANK	**GAPDH**	**GAPDH**
14	**PPIA**	**PPIA**	**PPIA**	**PPIA**	**RPL13A**	**RPL13A**	**ACTB**	**ACTB**

Figure 4.6d. Semi quantitative analysis of mRNA changes after exposure to wild type TIMP-1 relative to values obtained from T2G N TIMP-1 array.. For simplicity, the "fold change" in spot intensity is expressed as a colour: BLACK: no signal, RED: no change, GREEN: 2-10 fold increase, BLUE: greater than 10 fold increase. Genes inhibiting apoptosis (-), promoting apoptosis (+).

4.8 Effect of TIMP-1 on FAS/APO-1/CD95 and Fas ligand.

The cleavage and release of the pro apoptotic ligand Fas ligand has been demonstrated to be MMP dependent (Krammer 2000; Tanaka et al. 1996). Moreover, it has been suggested that Fas/Fas ligand may regulate HSC apoptosis (Saile et al. 1997; Gong et al. 1998; Saile et al. 1999). Further experiments were undertaken to determine whether TIMP-1 regulated Fas ligand cleavage in human HSC. HSC were incubated for 18 hours in conditions of absolute serum deprivation with BSA or BSA with TIMP-1 (142.5ng/ml). HSCs proteins were extracted and supernatants collected. After normalising for cell number (by DNA concentration using the Picogreen technique) these extracts were analysed by ELISA for Fas and Fas ligand as described in the methods. TIMP-1 treatment of human HSC had no effect on cellular Fas or Fas ligand protein levels compared to control cells treated with BSA alone. Supernatant Fas and Fas Ligand protein levels were undetectable in all experimental conditions (Figure 4.7a & 4.7b).

FIGURE 4.7A. EXPOSURE TO TIMP-1 DOES NOT AFFECT CELLULAR FAS LEVELS ON HUMAN ACTIVATED HSC.

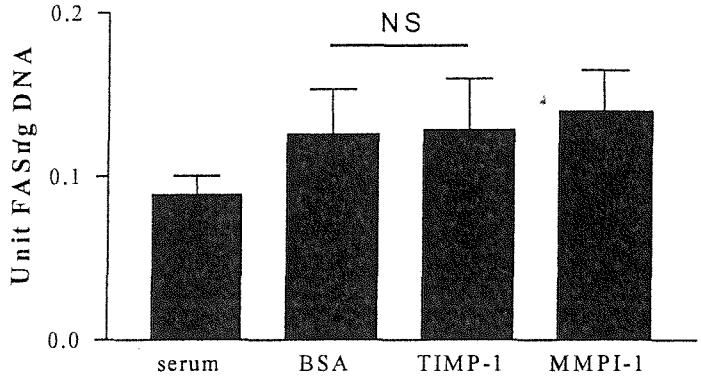


Figure 4.7a. To determine if TIMP-1 effected the level of cell surface Fas protein, human activated HSC were exposed to TIMP-1 (5nM) and the commercial MMP inhibitor MMPI-1 (1μM) for 18 hours. Cells extracts were made and normalised for cell number by Picogreen DNA assay then Fas protein was measured using a commercial ELISA as described in the methods section. MMP inhibition used in this experiment did not appear to change the levels of Fas. (Data shown are mean+/- SEM Units Fas per ng DNA, n=3)

FIGURE 4.7B EXPOSURE TO TIMP-1 DOES NOT AFFECT CELLULAR FAS LIGAND LEVELS IN HUMAN ACTIVATED HSC

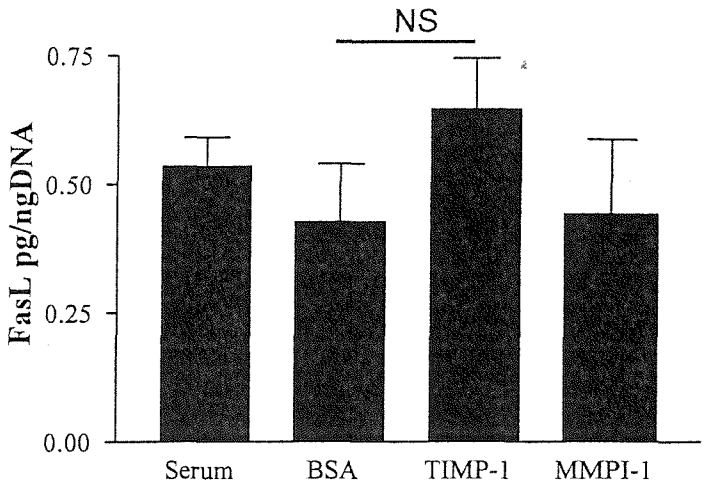


Figure 4.7b. To determine if TIMP-1 effected cell surface Fas Ligand, activated human HSC were exposed to TIMP-1 (5nM) and the commercial MMP inhibitor MMPI-1 (1μM) for 18 hours. Cell extracts were normalized for cell number by Picogreen DNA assay and then assayed for Fas Ligand using the commercial ELISA for Fas ligand as described in the methods section. MMP inhibition did not appear to effect cell surface Fas ligand. (Data expressed are mean+/-SEM Units Fas Ligand per ng DNA, n=3)

4.9 TIMP-1 enhances expression of Bcl-2 protein.

The protein Bcl-2 regulates the properties of cells to undergo apoptosis by intercalating into the mitochondria membrane (Hengartner 2000). The protein Bcl-2 increases the resistance of cells to apoptosis, in contrast Bax is pro apoptotic. To define changes in the protein level of Bcl-2 and Bax extracts from HSC treated with cycloheximide, in the presence and absence of 100ng/ml TIMP-1 for 18 hours, were analysed by Western blotting. Relative to cells treated with cycloheximide alone, cells treated with TIMP-1 and cycloheximide demonstrated maintained or enhanced levels of Bcl-2 protein expression (Figure 4.8a), which approached the levels observed in HSC maintained in serum alone. Parallel experiments were undertaken for the pro apoptotic protein Bax. TIMP-1 appeared to reduce Bax (Figure 4.8b).

FIGURE 4.8A. EFFECT OF TIMP-1 ON BCL-2 LEVELS IN ACTIVATED RAT HSCS TREATED WITH CYCLOHEXIMIDE MEASURED BY WESTERN BLOT.

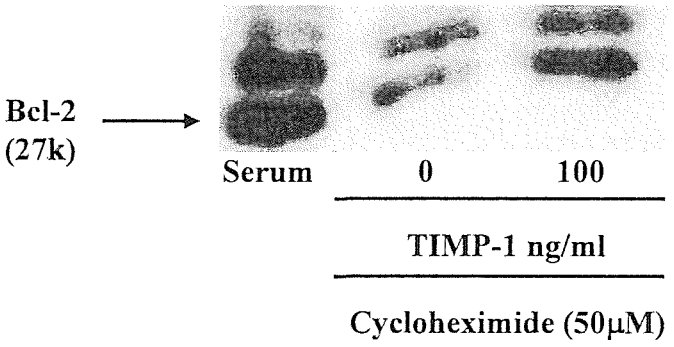


Figure 4.8a. Western blotting for Bcl-2 of equal quantities (10μg) of protein extracts from HSC exposed to serum alone, cycloheximide alone and cycloheximide with TIMP-1 protein (100ng/ml). Treatment with TIMP-1 resulted in increased in Bcl-2 expression relative to HSC treated with cycloheximide alone (n=3).

FIGURE 4.8B. EFFECT OF TIMP-1 ON BAX LEVELS IN ACTIVATED RAT HSCS TREATED WITH CYCLOHEXIMIDE MEASURED BY WESTERN BLOT.

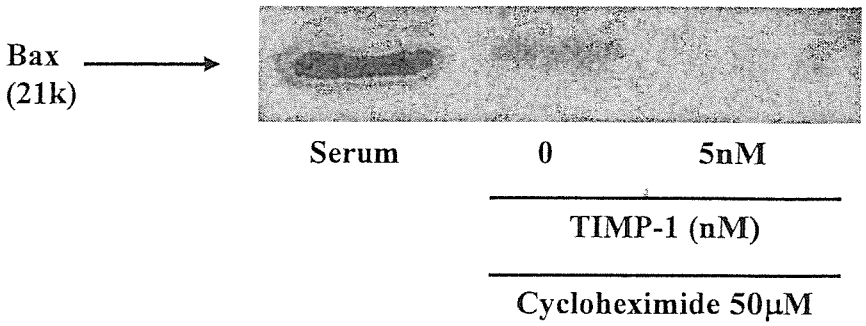


Fig 4.8b. Western blotting for Bax of equal quantities (determined by protein concentration) of protein extracts from HSC exposed to serum alone, cycloheximide alone and cycloheximide with TIMP-1 protein (100ng/ml). Treatment with TIMP-1 resulted in decreased Bax expression relative to HSC treated with cycloheximide alone (n=3).

4.10 Neither TIMP-1 nor TIMP-2 directly inhibit Caspase-3 activity

To exclude a direct effect of TIMP-1 or TIMP-2 on Caspase-3 activity recombinant human Caspase-3 (Calbiochem, Nottingham, UK) was incubated with TIMP-1 and TIMP-2 in varying concentrations (285-2850ng/ml and 210-2100ng/ml respectively) for 1 hour before Caspase-3 substrate was added to the reaction. Neither TIMP-1 nor TIMP-2 reduced Caspase-3 activity directly (Figure 4.9a & 4.9b).

FIGURE 4.9A. TIMP-1 DOES NOT DIRECTLY INHIBIT CASPASE-3 ACTIVITY

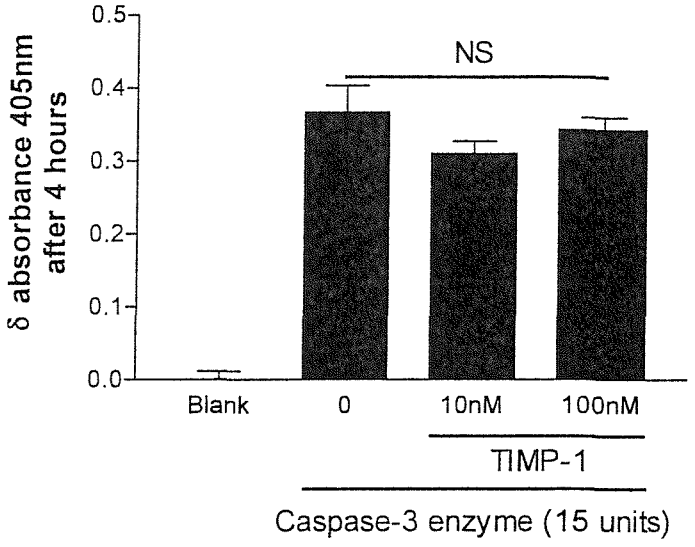


Figure 4.9a To determine if TIMP-1 directly effected Caspase-3 activity, recombinant Caspase-3 was incubated with varying concentrations of recombinant TIMP-1 (10-100nM) for 1 hour before Caspase-3 substrate was added to the reaction. No significant reduction in Caspase-3 activity was observed. (Data expressed are mean and standard error change in absorbance at 405nm after four hours)

FIGURE 4.9B TIMP-2 DOES NOT DIRECTLY INHIBIT CASPASE-3 ACTIVITY

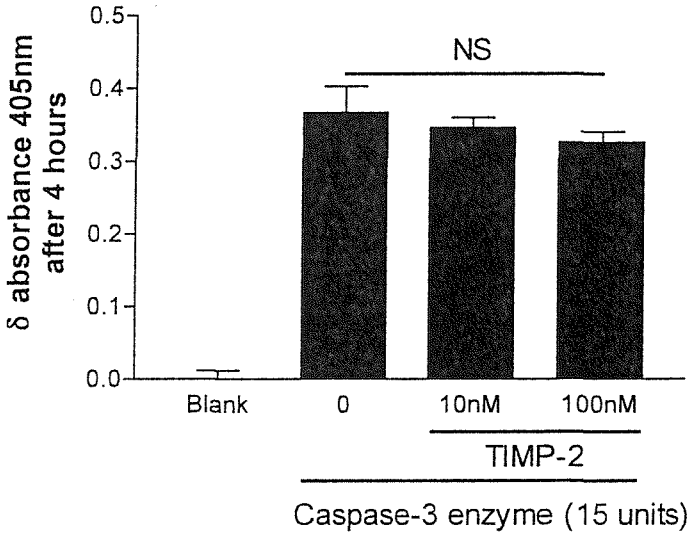


Figure 4.9 To determine if TIMP-2 directly effected Caspase-3 activity, recombinant Caspase-3 was incubated with varying concentrations of recombinant TIMP-2 (10-100nM) for 1 hour before Caspase-3 substrate was added to the reaction. No reduction in Caspase-3 activity was observed. (Data expressed are mean and standard error change in absorbance at 405nm after four hours).

4.11 The fate of TIMP-1 during apoptosis of human HSC

To determine the fate of TIMP-1 during apoptosis, human HSC were induced into apoptosis by exposure to gliotoxin. A time course was under taken with collection of conditioned media and cells for further analysis for TIMP-1 and MMP-2 by western blotting. Detectable levels of TIMP-1 were found in conditioned media and did not appear to change in concentration either before, during or after apoptosis. In contrast, western blotting for TIMP-1 demonstrated that there was a dynamic change in cellular TIMP-1 with time. Firstly, there was an increase in cellular TIMP-1 up to 6 hours, after this the level of TIMP-1 in the cell extracts appeared to decrease (Figure 4.10a & 4.10b). A bioinformatic study of the amino acid sequence for TIMP-1 revealed that human TIMP-1 was a potential substrate for active Caspase-3 enzyme (Figure 4.10c). Caspase-3 is known to be highly active during apoptosis of HSC and it is feasible that TIMP-1 trapped within apoptotic cells is degraded by caspase-3.

FIGURE 4.10A. TIME COURSE AND WESTERN BLOT FOR HUMAN TIMP-1 DURING APOPTOSIS INDUCED BY GLIOTOXIN

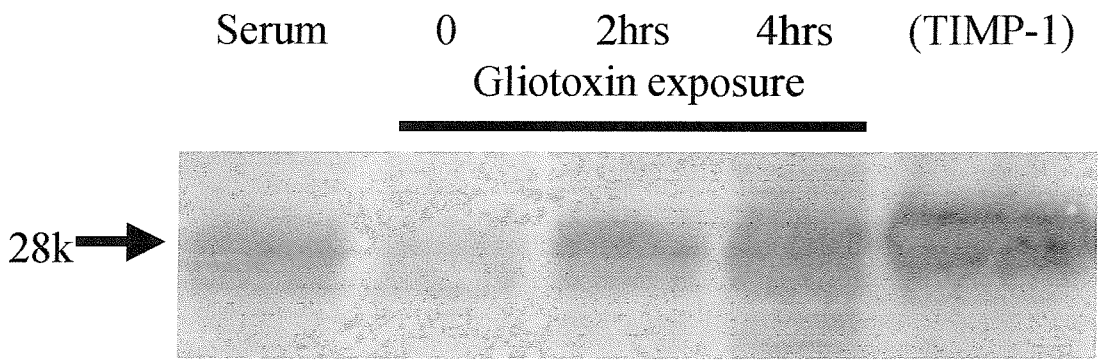


Figure 4.10a. To determine the fate of intracellular TIMP-1 during apoptosis a time course was undertaken following exposure of human HSC to gliotoxin (1.5μM) from 0 to 4 hours. Western blotting of cell extracts to determine intracellular TIMP-1 demonstrated detectable TIMP-1 within cells kept in serum for 4 hours. Incontrast, cells induced into apoptotosis by gliotoxin initially showed reduced TIMP-1 levels (compared to serum) that inclreased with time over a 0–4 hour period. (TIMP-1) is a recombinant positive control. Phase contrast observation showed a significant level of apoptosis associated with exposure to gliotoxin.

FIGURE 4.10B. TIME COURSE WESTERN BLOT FOR TIMP-1 AND MMP-2.

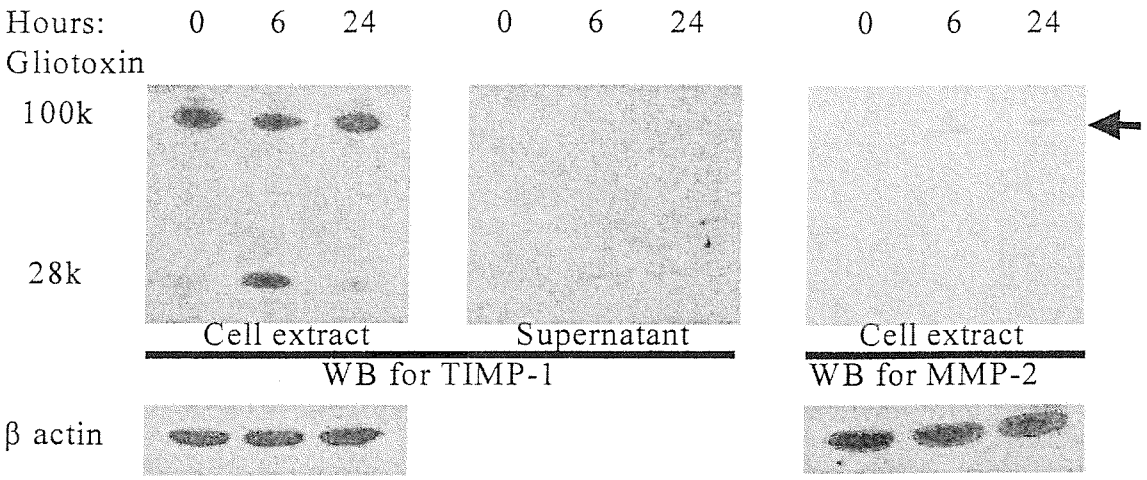


Figure 4.10b. A longer time course of apoptosis induced by gliotoxin was undertaken to examine the fate of TIMP-1 and MMP-2. Phase contrast observation showed clear evidence of apoptosis by morphology after 2-6 hours. Western blot for TIMP-1 (WB for TIMP-1) showed a slight increase from 0 to 6 hours which was then followed by a decrease at 24 hours. The TIMP-1 in the condition supernatant did not appear to change in quantity. A highly immunoreactive band was observed at 100k in the TIMP-1 western blot, a similar sized band was observed when the same cell extracts were blotted for MMP-2 (see arrow in WB for MMP-2). This band probably represents at least in part a complex of MMP-2 and TIMP-1.

FIGURE 4.10C. A BIOINFORMATIC STUDY OF HUMAN TIMP-1:

^{1}CTCVPPHPQTAFCNS **D** LVIRAKFVGTPEVNQTTLYQR

YEIKMTKMYKGFQALG **D** AA **D** IRFVYTPAMESVCGY

FHRSHNRSEEFLIAGKLQ **D** GLLHIT TCSFVAPWNSLS

LAQRRGFTKTYTVGCEECTVFPCLSIPCKLQSGTHCLW

T **D** QLLQGSEKGFQSRHLACLPREPGLCTWQSLRSQIA^{184}

Figure 4.10c. The amino acid sequence for mature human TIMP-1 was taken from a protein data base of Swiss Prot (http://us.expasy.org/sprot). Caspase-3 is known to be a promiscuous protease that cleaves peptides after aspartate residues (D in the figure). Five aspartate residues are present in human TIMP-1 suggesting that TIMP-1 is a potential substrate for Caspase-3.

4.12 TIMP-1 is not degraded by active caspase-3 in vitro

To determine if TIMP-1 is a substrate for caspase-3 TIMP-1 was entered into a substrate assay. There was no evidence that caspase-3 degraded TIMP-1 when assessed by SDS PAGE and western blotting for TIMP-1 (Figure 4.10d).

FIGURE 4.10D. SUBSTRATE CLEAVAGE ASSAY BETWEEN TIMP-1 AND ACTIVE CASPASE-3.

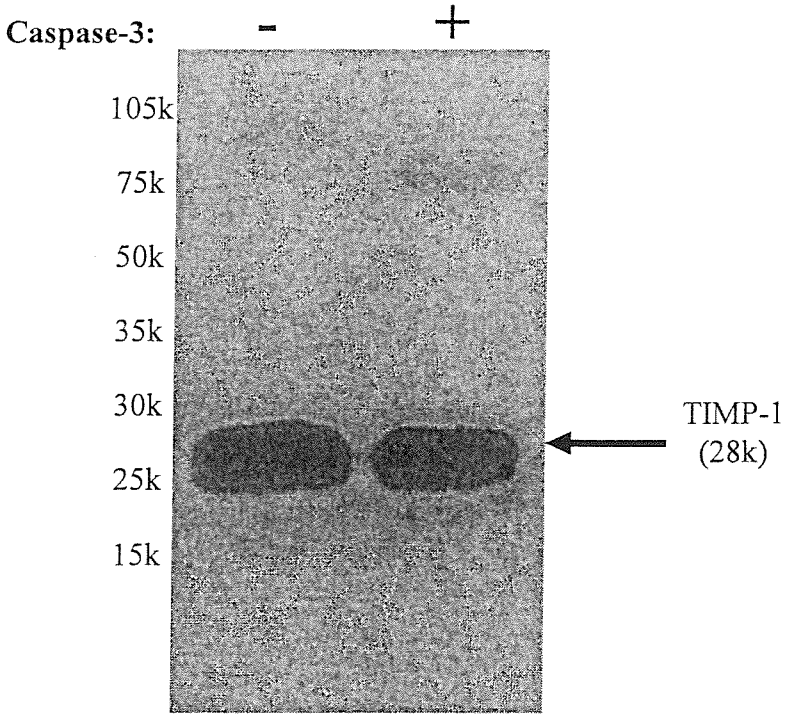


Figure 4.10d. To determine if TIMP-1 was degraded by active caspase-3, recombinant human TIMP-1 (25ng) was either incubated with assay buffer or active caspase-3 enzyme (20 I.U.) for 1 hour at 37°C then subjected to SDS PAGE and western blot with a polyclonal antibody to TIMP-1. There was no evidence that caspase-3 had degraded the TIMP-1. Data shown are representative of three separate experiments.

4.13 Summary of findings from mechanistic studies:

1. Inhibition of apoptosis of activated human and rat hepatic stellate cells by TIMP-1 is mediated via effects on MMP inhibition.
2. Apoptosis was inhibited by commercial inhibitor of MMPs.
3. Active MMP-2 promotes apoptosis of human HSC
4. Multiple apoptosis regulated genes are changed with exposure to TIMP-1
5. TIMP-1 does not effect Fas or Fas ligand in activated human hepatic stellate cells.
6. Bcl-2 was increased in HSC treated with TIMP-1.
7. Bax was reduced in HSC treated with TIMP-1.
8. Neither TIMP-1 nor TIMP-2 directly inhibits active caspase-3 enzyme.
9. In apoptotic HSC, there are dynamic changes in the level of cellular TIMP-1.

4.14 Discussion of mechanistic studies

4.14.1 The data described in this chapter provides strong evidence that the mechanism by which TIMP-1 inhibits apoptosis is via inhibiting MMP activity. Study of this mechanism was approached in a number of ways. Firstly, by using the published K_I of MMP inhibitors including TIMPs, commercial inhibitors and the T2G mutant inhibitor it was possible to guide further studies towards using active recombinant MMPs on cultured cells. Studies with the synthetic MMP inhibitor, MMPI-1, suggested that MMP inhibition was likely to be the mechanism mediating survival of HSC. This was confirmed by using the T2G mutant N-TIMP-1. These studies demonstrate directly that inhibition of apoptosis of HSCs by TIMP-1 is in fact mediated via its effects on MMP activity. The T2G mutant N-TIMP-1 protein differs from the wild type N-TIMP-1 protein by only a single amino acid substitution (Threronine to Glycine at amino acid position number 2) which reduces the inhibition constant of TIMP-1 for MMP-1, MMP-2 and MMP-3 by factors of 6000, 93,000 and 700 respectively(Meng et al. 1999). Thus T2G mutant N-TIMP-1 is particularly poor at inhibiting active MMP-2. This indicates that active MMP-2 is most likely MMP to be involved in apoptosis of cultured human HSC.

4.14.2 Studies with the commercial MMP inhibitors confirmed further that MMP inhibition alone could reduce apoptosis induced by cycloheximide. A potential criticism of this work is that the true "selectivity" of these inhibitors may not be that precise. Indeed, the fact that all of the MMP inhibitors appear to reduce apoptosis suggests that none are particularly selective.

The most compelling evidence of active MMP-2 involvement in apoptosis is the data on human cells directly exposed to high concentrations of active MMP-2. This clearly produced significant apoptosis that could not be matched by active MMP-3 or MMP-9. Interestingly, the T2G mutant is a poor inhibitor of MMP-2 and this further points towards MMP-2 being involved in human HSC apoptosis. Given the unique regulatory features of MMP-2 including a functioning p53 motif in its gene promoter(Bian and Sun 1997) and a complex cell surface activation involving MT1 MMP(Strongin et al. 1995), MMP-2 is clearly a good candidate MMP to study in greater depth and these studies are described in chapter 5.

4.14.3 Studies of the effects of TIMP-1 on apoptosis related genes revealed that multiple different genes were increased with MMP inhibition. This was not born out in the case of Fas or Fas ligand where TIMP-1 did not appear to change the levels of these proteins in cell extracts. In contrast, appropriate changes in bax (decreased) and bcl-2 (increased) suggest that the anti apoptotic effect of TIMP-1 may be explained at least in part by changes in these apoptotic regulatory proteins. The large number of genes that appear to change in the gene array suggest that MMP inhibition may play a key role in the apoptotic phenotype of human HSC. This would fit well with the model of HSC defaulting to apoptosis unless survival signals are maintained. This model is further tested in chapter 5 which focuses on cell matrix survival signals and chapter 6 which focuses on cell to cell survival signals.

4.14.4 The potential mechanisms through which apoptosis may be regulated by TIMP-1 are legion and may involve more than one MMP. A major candidate mechanism through which TIMPs mediate survival is by preventing matrix degradation. HSC may gain direct signals from matrix. Moreover, matrix contains numerous matrix bound cytokines that may have anti-proliferative and/or pro-apoptotic effects on local cell populations. (for example TGF beta) that may be liberated by matrix degradation. In the context of the liver fibrosis recovery model (Iredale et al. 1998), during the degradation of fibrotic tissue, release of matrix bound cytokines may also be important in determining the pattern of recovery and apoptosis of activated HSCs. If TIMP-1 reduces apoptosis via preventing matrix degradation, it may do this by preventing MMP degradation of some key targets. Firstly, release of matrix bound pro apoptotic factors would be prevented. Secondly, intact matrix may provide direct cell survival signals and present matrix bound survival signals in a spatially effective manner. TIMP would preserve such signals. In support of this hypothesis, a mutant collagen resistant

to collagenase digestion, has been demonstrated to promote HSC survival in models of fibrosis (Issa et al. 2003). Moreover, the in vivo studies described in chapter 3 are compatible with TIMP-1 promoting HSC survival through MMP inhibition and protection of the fibrotic matrix.

4.14.5 There are further mechanisms by which MMP inhibition may mediate survival in vivo. It is known that many cell surface proteins can be cleaved provided their appropriate "sheddase" is present and active. In cases where MMPs mediate shedding of receptors (e.g. TNF receptor) TIMPs may indirectly regulate cell behaviour. Recently TIMP-3 has been demonstrated to induce apoptosis in human colonic carcinoma cells by stabilizing TNF alpha receptors on the cell surface (Smith et al. 1997). Endothelial cells have been demonstrated to shed receptors for tumour necrosis factor following induction of apoptosis which may be a mechanism to limit inflammation in response to apoptotic cell death (Madge et al. 1999). A further MMP dependent cell surface protein system regulating apoptosis is the Fas/Fas ligand system. Hepatic stellate cells are known to express Fas and Fas Ligand on their cell surface (Saile et al. 1997; Gong et al. 1998). TIMP-1 did not have any effect on cellular Fas or Fas ligand protein levels in activated human hepatic stellate cells. It is also possible that TIMP-1 might inhibit apoptosis by preventing the shedding of a pro-survival receptor, for example Insulin like growth factor-1 receptor, which is known to prevent apoptosis in activated hepatic stellate cells and related cells (Baker et al. 1994; Issa and Williams 2001). A further MMP cleaved cell surface receptor that regulates cell survival is Cadherin. The Cadherin and beta catenins pathway is known to impact on cellular Bcl-2 levels and thus the inherent tendency for a given cell to undergo apoptosis (Herren et al. 1998).

4.14.6 Together with previous studies, the data described in this thesis so far provide strong evidence that TIMP-1 is mechanistically important in promoting fibrosis: Firstly by directly inhibiting MMPs thus promoting matrix accumulation, and secondly, by inhibiting the apoptosis of activated hepatic stellate cells.

Chapter 5

5. Studies of the role of MMP-2 in HSC apoptosis

5.1 Introduction

5.1.1 The role of the hepatic stellate cell in the development and spontaneous resolution of liver fibrosis can be considered a paradigm for solid organ fibrosis and wound healing in the body. An emerging concept from studies of spontaneous resolution of experimental fibrosis, including experimental liver fibrosis, is the observation that there is a decrease in the fibrogenic cell population mediated by apoptosis (Clark 1993; Duffield et al. 2000; Iredale et al. 1998; Issa et al. 2001). In each example, apoptosis accompanies evidence of matrix degradation and evidence of matrix degrading metalloproteinase activity. In contrast, during progressive fibrosis, the presence of excess TIMPs inhibits MMP activity and prevents matrix degradation. Moreover, over expression of TIMP-1 results in increased fibrosis and a failure of spontaneous resolution of fibrosis in vivo (Yoshiji et al. 2000). Recent data have suggested that TIMPs may also promote survival of both fibrogenic cells and malignant cells (Guedez et al. 1998; Lin et al. 2002). The work I have described in the previous chapters has shown that inhibition of HSC apoptosis by TIMP-1 is mediated via inhibition of MMP activity.

5.1.2 Taken together, these data suggest that MMP activity may promote or facilitate apoptosis of fibrogenic cells (including HSC) during resolution of tissue fibrosis. It is self evident that the specific MMP mediating or facilitating apoptosis must be expressed during the switch from established fibrosis to resolving fibrosis. Previous studies have demonstrated in a series of animal and human models that MMP-2 satisfies this criterion, being expressed in advanced fibrosis and being detectable in the livers of fibrosis undergoing resolution (Benyon et al. 1995; Benyon et al. 1999). Moreover, MMP-2 has recently been demonstrated to be activated by HSC as they undergo apoptosis (Preaux et al. 2002). These data point for a potential role for MMP-2 in providing an extra-cellular mechanism that mediates or facilitates HSC apoptosis.

5.1.3 Type I collagen is a major product of activated HSC (Maher et al. 1988) and its presence in fibrotic liver correlates with the presence of activated HSC (Iredale et al. 1998). Recently, using mice bearing a mutated collagen-I gene (r/r mice) (Krane et al. 1996), which confers resistance to degradation by MMPs, collagen degradation was shown to be a critical factor promoting HSC apoptosis during spontaneous recovery from experimental liver fibrosis (Issa

et al. 2003). In this study by Issa et al. liver injury of rats was undertaken with 8 weeks of carbon tetrachloride injections. During the following 28 days of spontaneous recovery, the liver collagen content significantly decreased in the wild type mice but remained elevated in r/r mice. Furthermore, HSC apoptosis was observed to be reduced in the r/r mice compared to wild type controls. This data suggests that type I collagen promotes the survival of activated HSC in vivo and is thus an obvious MMP substrate to study with regard HSC survival or apoptosis in tissue culture models. Because the mutant type I collagen extracted from the mouse tail is resistant to degradation from a number of MMPs including MMP-2, r/r collagen provides a novel method of dissecting out the potential role that matrix turnover has on cell survival or apoptosis.

5.1.4 In the following experiments I have addressed the hypothesis that active MMP-2 mediates or facilitates HSC apoptosis by providing alterations in extracellular signals. Emphasis has been placed on the role of type I collagen acting via cell-matrix interactions because it is a described substrate for active MMP-2 (Aimes and Quigley 1995).

5.2 MMP-2 in spontaneous recovery from liver fibrosis

Apoptosis of HSC during resolution of fibrosis is associated with increased liver expression of active MMP-2. Previous studies have shown that HSC population decreases during spontaneous resolution of experimental liver fibrosis by apoptosis in the carbon tetrachloride and bile duct ligation experimental models of liver fibrosis (Iredale et al. 1998; Issa et al. 2001). Apoptosis coincides with decreases in hepatic expression of MMP inhibitor TIMP-1 and plasminogen activator inhibitor expression (Iredale et al. 1998; Zhang et al. 1999). Previous studies described in chapter 3 & chapter 4 have also identified TIMP-1 as a survival factor for HSC, which suggests that one or more MMPs might promote HSC apoptosis. Therefore, the expression of MMP-2 protein was examined during spontaneous resolution of liver fibrosis using gelatin zymography. Gelatin zymography of whole liver homogenate during the 28 days of fibrosis resolution after six weeks of carbon tetrachloride administration demonstrated 72kDa proMMP-2 but in addition the 62kDa active form of MMP-2 was apparent. Therefore, activation of MMP-2 temporally correlated with loss of HSC by apoptosis in this tissue model as previously reported (Iredale et al. 1998) (Figure 5.1).

FIGURE 5.1. GELATIN ZYMOGRAPHY OF WHOLE LIVER HOMOGENATE FROM PERIOD OF SPONTANEOUS RECOVERY FROM LIVER FIBROSIS.

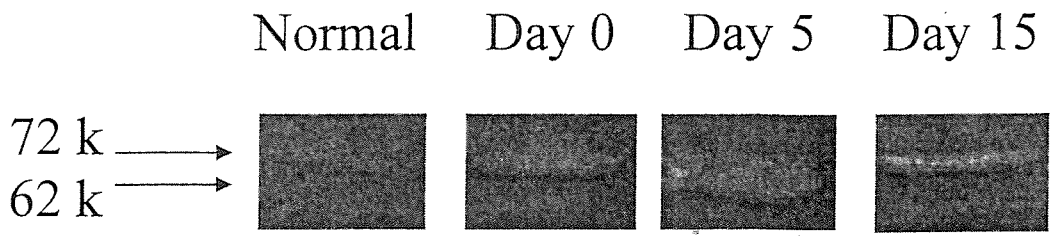


Figure 5.1. Liver fibrosis was induced in rats by carbon tetrachloride administration for 6 weeks. Liver homogenates were prepared from normal rats, at peak fibrosis (Day 0), and at 5 and 15 days after the last injection of CCl_4, during resolution of fibrosis. Equal protein aliquots were subjected to gelatin zymography for detection of 72kD pro-MMP-2 and 62kD activated MMP-2. Figure shows representative zymogram from 4 independent liver homogenates at each time point. Zymography was undertaken by Dr Xiaoying Zhou.

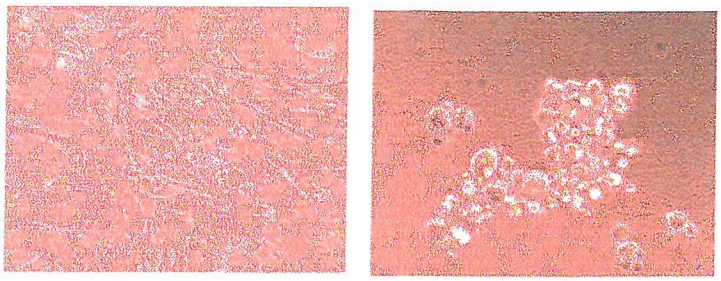
5.3 Apoptosis of HSC in culture is associated with proMMP-2 activation

MMP-2 activity in HSC conditioned media was measured using the Biotrak MMP-2 activity assay as described in the materials and methods. Also, gelatin zymography of human HSC conditioned media was undertaken. Conditioned media from human HSC that had been washed three times in serum free media and then left overnight contained detectable amounts of 72kDa proMMP-2 as described in previous reports (Benyon et al. 1999). Cells exposed to gliotoxin developed apoptotic morphology within 6 hours when assessed by phase contrast microscope or acridine orange staining as previously described (Wright et al. 2001) (Figure 5.2a & 5.2b). Measurement of active MMP-2 in conditioned media using the Biotrak activity assay demonstrated that there was a significant increase in concentration of active MMP-2 over the initial 6 hour period of apoptosis (Figure 5.2c). Furthermore, this increase in active MMP-2 was dependent on caspase active as parallel experiments with a caspase inhibitor (zVADfmk) did not show increases in active MMP-2 after exposure to gliotoxin for four hours (Figure 5.3d).

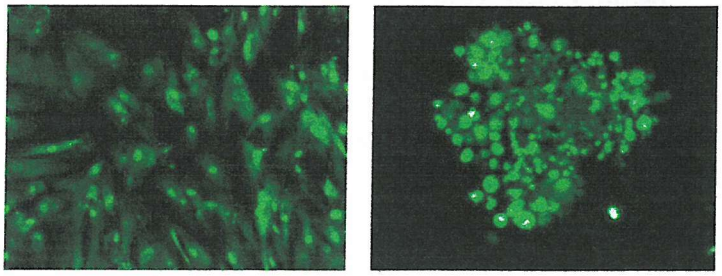
FIGURE 5.2. APOPTOSIS OF HUMAN HSC AND ACTIVATION OF MMP-2

Gliotoxin hours exposure: **0 hours:** **6 hours:**

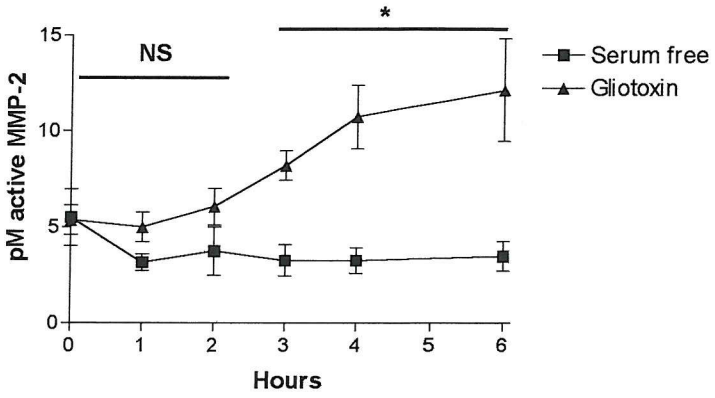
A. Phase contrast microscopy



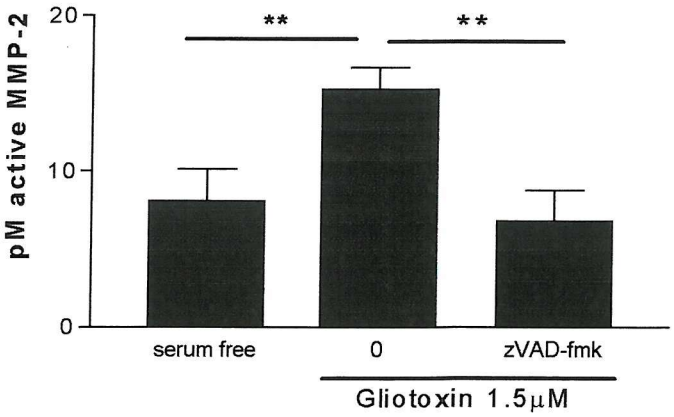
B. Acridine orange staining



C. Active MMP-2 assay



D.



6.11 Human HSC express N-cadherin at the cell membrane with beta catenin

Staining for N-Cadherin intracellular moeity demonstrated that in human HSC N-Cadherin is located at the cell surface and at the cell surface membrane as would be expected. In addition, parallel staining demonstrated that beta catenin was also localised mainly to the cell membrane (Figure 6.11).

FIGURE 6.11. STAINING FOR N-CADHERIN AND BETA CATENIN IN HUMAN HSC.

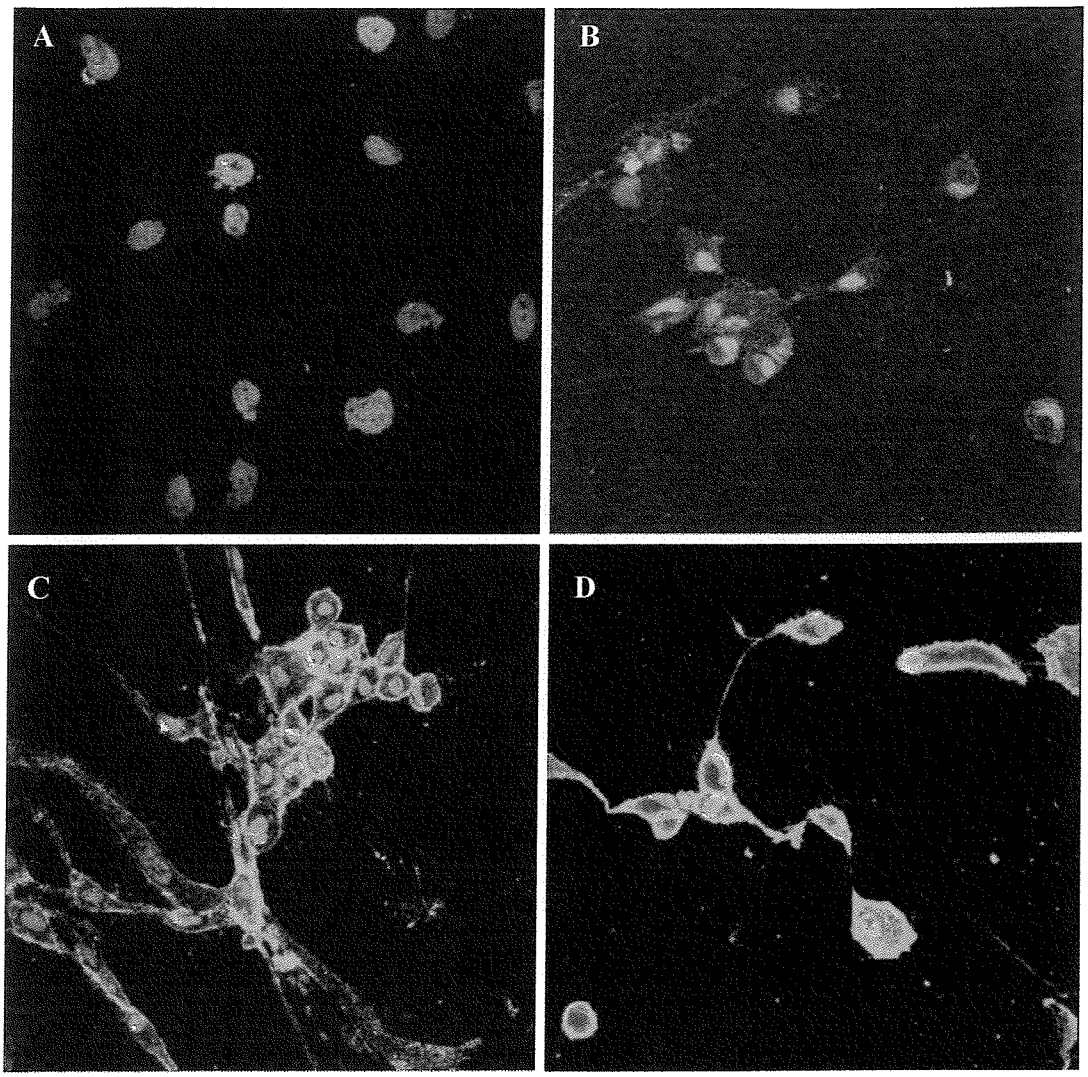


Figure 6.11. Laser confocal imaging Z series of human HSC cultured in serum containing media. A and B are negative controls treated without a primary antibody and stained with 7-actinomycin-D to show nuclei. C: Staining for N-cadherin (intracellular epitope) reveals a membrane location for N-cadherin. D: Staining for beta catenin reveals its location is also closely associated with the cell membrane. (Images are representative of two separate experiments, magnification x40).

Figure 5.2. A. Phase contrast pictures (magnification x 40) of human HSC before and after exposure to gliotoxin. Gliotoxin clearly induced an apoptotic morphology. B. Acridine orange staining under blue fluorescence before and after exposure to gliotoxin demonstrated that apoptotic cells are rounded with evidence of nuclear condensation and fragmentation. C. Conditioned media from apoptotic human HSC showed increased levels of active MMP-2 (Data shown are mean and SEM, NS 0-2 hour time points compared, *$p<0.05$ for 3-6 hour time points compared by Student's t test, n=3).. D. In parallel experiments with zVAD fmk (a caspase inhibitor), there was no increase in active MMP-2 after exposure to gliotoxin for four hours in cells that were exposed to the caspase inhibitor (Data are mean and SEM from three separate experiments with different human cell preparations, **$p<0.005$ by Student's t-test, n=3). Active MMP-2 was measured using the Biotrak MMP-2 activity assay.

5.4 Active MMP-2 promotes proliferation of human HSC in culture

To determine the effect of active MMP-2 on proliferation, cultured human HSC were exposed to a range of concentrations (0-10nM or 0-620ng/ml) of active MMP-2 and proliferation quantified as described in chapter 2. The active MMP-2 was verified in size and active status by gelatin zymography (Figure 4.5a). Active MMP-2 had a significant mitogenic effect in low serum conditions at low concentration (0.01-1 nM or 0.62-62.0ng/ml). At a higher concentration (10nM) the mitogenic effect was diminished and no longer significant. This suggested that there was a bell shaped dose response (Figure 5.3). If active MMP-2 was co incubated with another mitogen for example PDGF or IGF-1 there was a significant increase in proliferation but there was no longer any evidence of an influence by the active MMP-2 independent of active MMP-2 concentration (data not shown).

FIGURE 5.3. EFFECT OF RECOMBINANT HUMAN ACTIVE MMP-2 ON HSC PROLIFERATION.

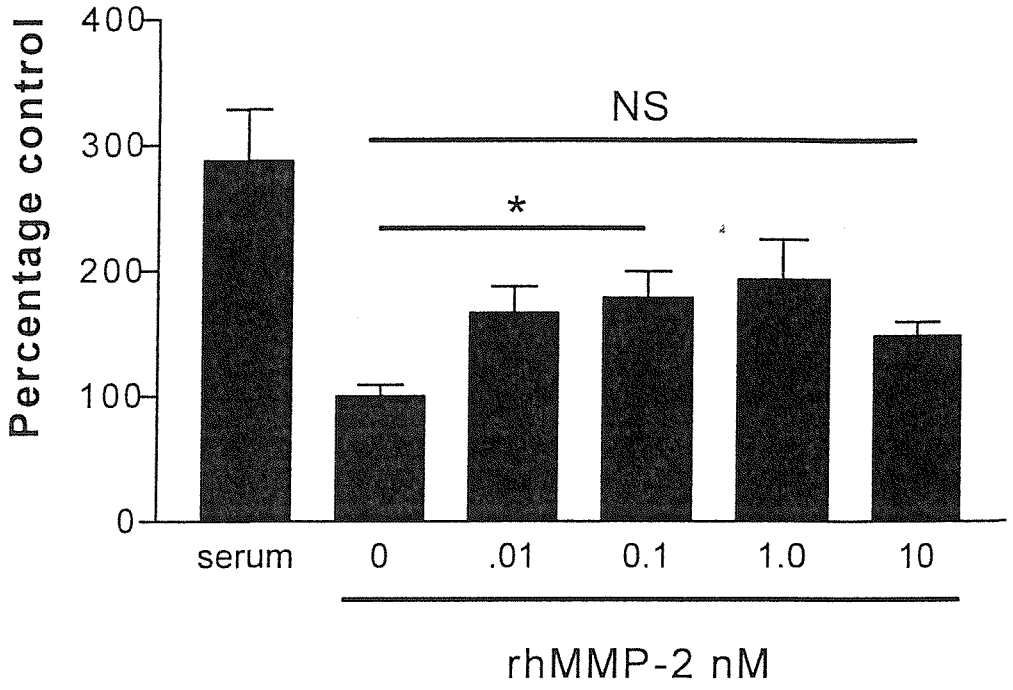


Figure 5.3b. Cultured HSC were exposed to recombinant human active MMP-2 over a concentration range of 0-10nM in serum free conditions. Low concentrations (0.01-0.1nM) of active MMP-2 produced a modest (50%) increase in HSC proliferation compared to untreated control HSC. At higher concentrations (10nM), active MMP-2 did not significantly increase cell proliferation. HSC were exposed to conditions for 18 hours in total and tritiated thymidine added after 6 hours from start of the incubation. (Data are mean ± SEM expressed as percentage untreated control cells which have been given the arbitrary value of 100%. NS not significant, *,p=0.03 by Student's t-test, n=4).

5.5 Active MMP-2 promotes apoptosis of human HSC

As the above studies in the rat model and HSC cultures implicated MMP-2 in HSC apoptosis, active MMP-2 was added directly to HSC cultured on plastic. HSC exposed to 10nM of recombinant active MMP-2 in serum free conditions with and without cycloheximide for four hours at 37°C. Whereas lower concentrations of active MMP-2 (2nM) had no significant effect on HSC apoptosis, a higher concentration (10-20nM) resulted in a significant increase in HSC apoptosis compared to medium containing MMP buffer alone when assessed by acridine orange (Figure 4.5b, chapter 4). Parallel experiments showed that the pro apoptotic effect of MMP-2 could be significantly reduced by co-incubation with TIMP-1, TIMP-2 or a selective inhibitor of MMP-2 (Figure 5.4).

FIGURE 5.4. EFFECT OF MMP INHIBITORS ON APOPTOSIS OF CULTURED HUMAN HSC INDUCED BY EXPOSURE TO ACTIVE MMP-2.

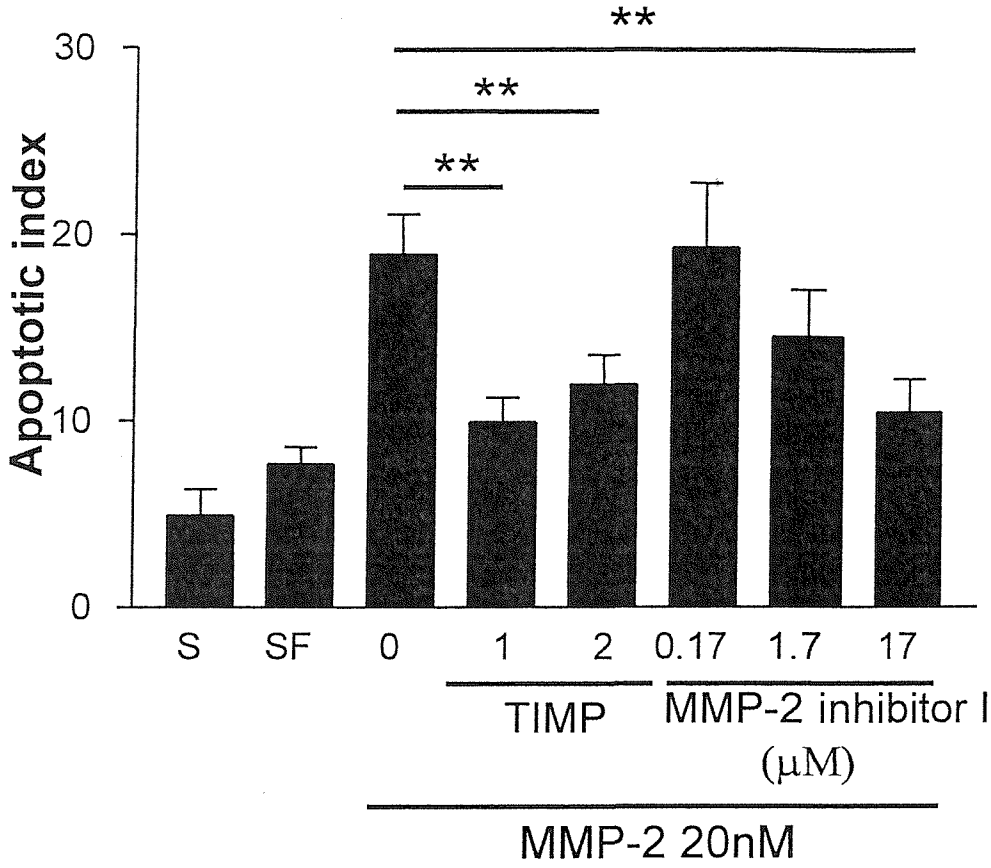


Figure 5.4. Cultured HSC were exposed for 4 hours to 20nM active MMP-2 to promote apoptosis in the presence or absence of MMP inhibitors including TIMP-1 (20nM), TIMP-2 (20nM) and the selective MMP-2 inhibitor MMP-2 inhibitor I. (Data are expressed as mean ± SEM of apoptotic index. **,$p<0.005$ by t-test, n=3).

5.6 Human HSC treated with active MMP-2 have increased Caspase-3 activity

Caspase-3 is a key enzyme involved in the apoptotic cascade and its activity has been used as a means of quantifying apoptosis (Hengartner 2000). To support the observations of MMP-2 induced apoptosis made by acridine orange staining (Figure 4.5b), parallel experiments were undertaken and Caspase-3 activity in cell lysates was assessed. HSC exposed to 10nM of active MMP-2 had significantly higher Caspase-3 activity compared to cells treated with MMP buffer alone (Figure 5.5). Addition of proMMP-2 had negligible effect on apoptosis, suggesting that the pro-apoptotic activity of MMP-2 observed in our experiments were due to MMP catalytic activity.

FIGURE 5.5. HUMAN HSC TREATED WITH 10NM OF ACTIVE MMP-2 HAVE INCREASED CASPASE-3 ACTIVITY.

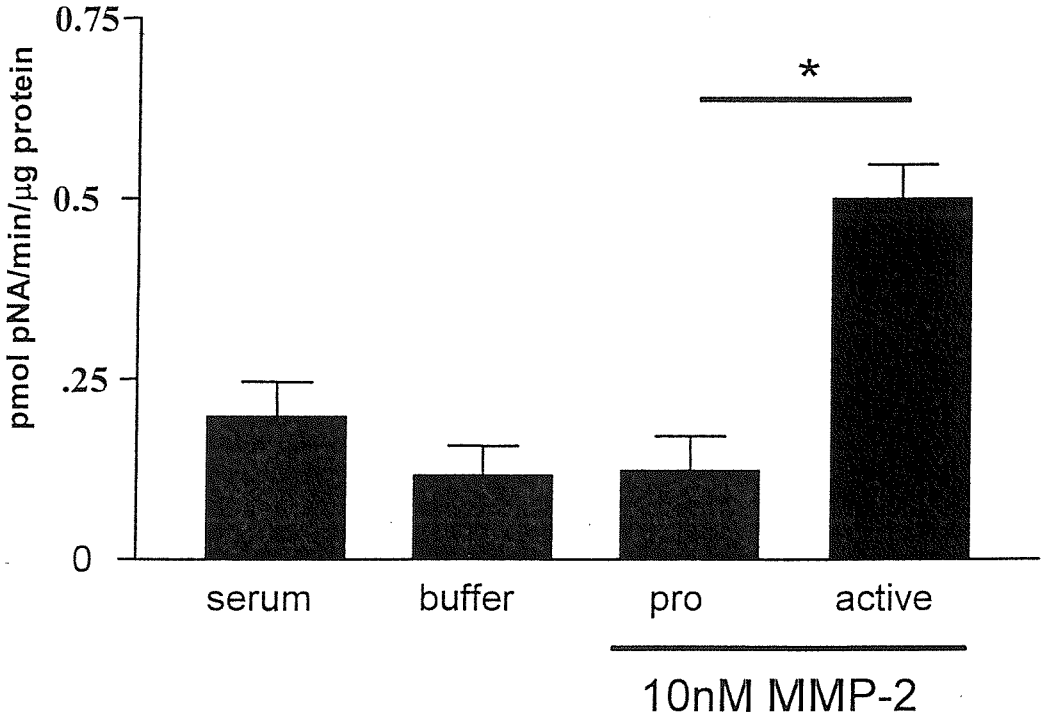


Figure. 5.5 To verify data from acridine orange counting, parallel experiments were undertaken using caspase-3 activity as another measure of apoptosis. Caspase-3 activity in HSC cell extracts were sigificantly higher in those treated with 10nM active MMP-2 compared to cells exposed to MMP buffer alone or pro MMP-2. (*$p<0.05$ by Student t test, n=3).

5.7 A selective MMP-2 inhibitor reduces apoptosis in HSC following induction of apoptosis by cycloheximide

Previous data described in chapters 3 and 4 showing that exogenous TIMP-1 and TIMP-2 inhibit apoptosis suggest that endogenous MMP activity plays a role in HSC apoptosis. Further studies therefore examined the effect of a synthetic selective MMP-2 inhibitor on HSC apoptosis induced by cycloheximide. Techniques of acridine orange staining and examination of nuclear morphology (Figure 4.4a) and Caspase-3 activity assay (Figure 5.6) both confirm that the selective MMP-2 inhibitor I (see Table 4.1 for details) caused a significant reduction in apoptosis induced by exposure to cycloheximide. This suggested that HSC derived active MMP-2 plays a role in HSC apoptosis.

FIGURE 5.6. CASPASE –3 ACTIVITY IN HSC EXTRACTS REDUCED BY EXPOSURE TO SELECTIVE MMP-2 INIHIBITOR.

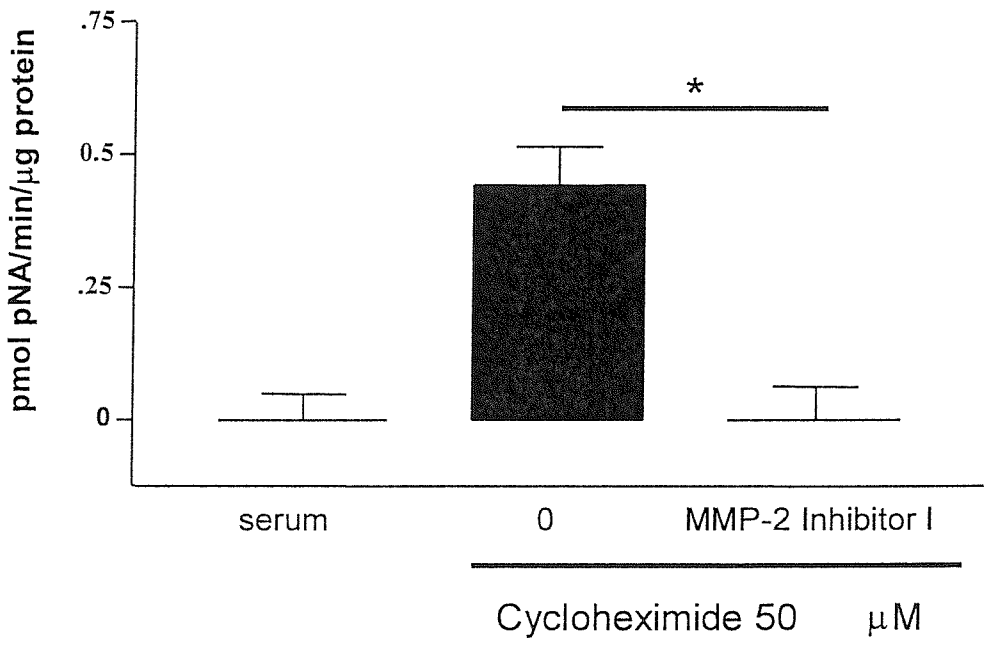


Figure 5.6b. Caspase-3 activity of cell extracts treated with synthetic selective MMP-2 inhibitor I and cycloheximide were compared with cells treated with cycloheximide alone. MMP-2 inhibitor I (1.7µM) significantly reduced Caspase-3 activity in HSC compared to cells treated with cycloheximide alone. (Data are expressed as mean ± SEM of Caspase-3 activity in pmol pNA/minute/ug protein and is representative of three separate experiments, *$p<0.05$).

5.8 MMP resistant type I collagen protects HSC from MMP-2 induced apoptosis.

Mice bearing the r/r collagen mutation have type I collagen which has been rendered non-degradable by interstitial collagenases and MMP-2 (Krane et al. 1996). Previous studies show that r/r mice have similar numbers of activated HSC in their livers compared to normal mice following 8 weeks CCl_4 treatment. However, cessation of CCl_4 dosing leads to resolution of fibrosis over 4 weeks and pronounced apoptosis of HSC in normal mice, in r/r collagen mice both collagen and HSC persist (Issa et al. 2003). This suggests that intact type I collagen protects HSC from apoptosis in vivo. MMP-2 is known to be able to degrade a number of matrix components, including type I collagen (Aimes and Quigley 1995). HSC grown on normal type I collagen were also prone to apoptosis when exposed to 10nM of active MMP-2 (Figure 5.7). However, HSC plated on r/r collagen had a significantly decreased apoptotic response to the active MMP-2 (Figure 5.7). This suggests that MMP-2 may regulate HSC apoptosis via degradation of type I collagen.

FIGURE 5.7. PRO APOPTOTIC EFFECT OF ACTIVE MMP-2 IS REGULATED BY SUBCELLULAR MATRIX TURNOVER.

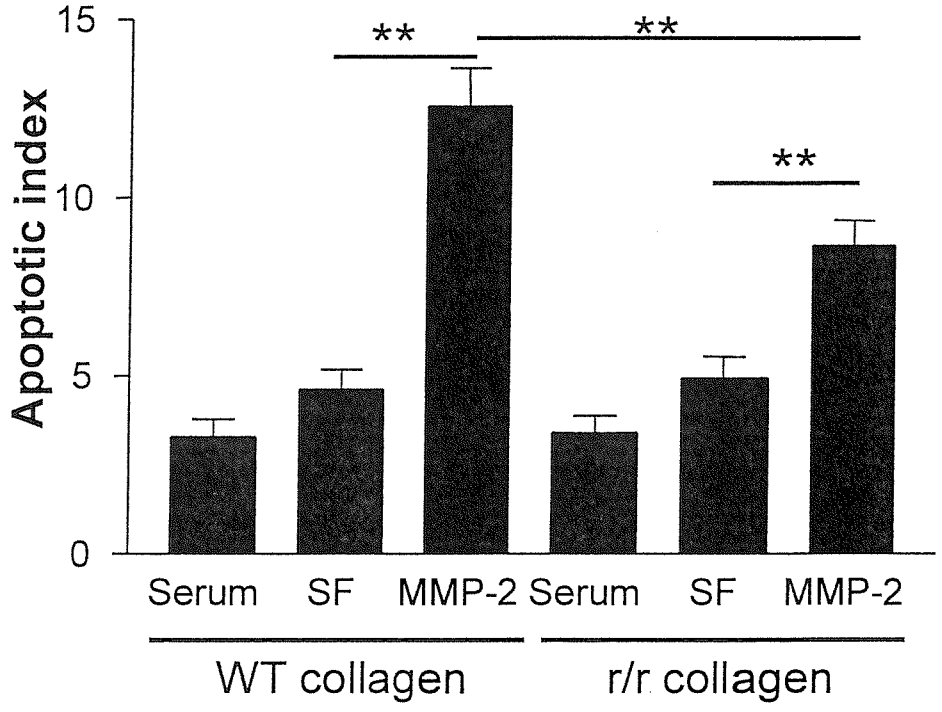


Figure 5.7. To determine if type I collagen subcellular matrix turnover affected the proapoptotic activity of active MMP-2 activated HSC were plated onto wild type collagen I (WT) or a mutated collagen type I (r/r) that is resistant to degradation by MMP-2. Cells were cultured on the two different collagens overnight before being

exposed to 10nM of active MMP-2 for 4 hours. Cell plated onto the wild type collagen were more sensitive to the proapoptotic stimulus of active MMP-2 compared to HSC plated on to mutant non degradable collagen. Apoptosis was assessed by acridine orange staining and cell counting. (Data are expressed as mean ± SEM apoptotic index. **,p=0.005 by student's T test, n=3).

5.9 Summary of the studies of the role of MMP-2 in HSC apoptosis:

1. Gliotoxin induced apoptosis of human HSC is associated with significant activation of MMP-2 that is detectable in conditioned media from apoptotic HSC.
2. The increase in active MMP-2 appears to be dependent on caspase activation as an inhibitor of the caspase cascade completely inhibited the increase of active MMP-2 seen follow exposure to gliotoxin.
3. In serum free conditions, active MMP-2 is a mitogen for human HSC at low concentrations (0.01-1.0nM).
4. Active MMP-2 promoted apoptosis of human HSC at higher concentrations (10-20nM) while pro MMP-2 had no affect on apoptosis.
5. The proapoptotic effect of active MMP-2 could be inhibited by TIMP-1, TIMP-2 or the selective MMP-2 inhibitor.
6. The proapoptotic effect of active MMP-2 is mediated at least in part via degradation of type I collagen.

5.10 Discussion

5.10.1 During spontaneous resolution of liver fibrosis there is loss of HSC by apoptosis which correlates with a transient increase in matrix metalloproteinase activity (Iredale et al. 1998). During the recovery the levels of MMPs appear to remain relatively constant while their natural inhibitor TIMP-1 decreases. The previous work described in earlier chapters has demonstrated that TIMP-1 inhibits apoptosis of HSC via mechanisms mediated via effects on MMP inhibition. The data I have described in this chapter has demonstrated that during spontaneous resolution of liver fibrosis there is activation of MMP-2. Furthermore, in vitro stimulation of HSC apoptosis by gliotoxin is associated with activation of MMP-2. To compliment these observations, the data demonstrate that exogenous active MMP-2 can promote apoptosis of human HSC in vitro. Moreover, this functional data provides an explanation for the correlation observed between the activation of MMP-2 and loss of HSC by apoptosis in vivo after the withdrawal of a toxic stimulus and spontaneous resolution of liver fibrosis. Overall this data provides cogent evidence that TIMPs regulate HSC survival by protecting survival signals gained from cell-matrix interactions while active MMP-2 is able to disrupt these survival signals directly in the extracellular milieu by degrading the molecules involved.

5.10.2 Anoikis is defined as apoptosis that is induced by inadequate or inappropriate cell-matrix interactions (Frisch and Screaton 2001). Adhesion to an appropriate matrix is required for survival in a variety of cells including HSC. Cells interact with matrix through a range of cell surface integrins. Disrupting cell matrix adhesion via disintegrin reagents promotes apoptosis, for example anti beta 1 integrin antibody promotes apoptosis of colonic epithelial cells (Strater et al. 1996) also RGD peptides that mimic matrix binding sites of integrins have been shown to promote apoptosis of HSC (Iwamoto et al. 1999). Anoikis may also be promoted by removing the matrix proteins themselves. Experimental induction of matrix degradation has been studied in transgenic mice with a range of proteolytic levels. In mammary gland epithelial cells of transgenic mice that produce an autoactivated MMP-3 (Stromelysin-1), epithelial cells under went unscheduled apoptosis during pregnancy. In contrast, the same strain of mice that had been bred to also have an excess of TIMP-1 did not show enhanced apoptosis (Alexander et al. 1996). This study indicates that to some extent changes in the balance between MMP and TIMP affects cell fate. Our data indicate similar roles for MMP and TIMPs either facilitating or preventing apoptosis respectively. Moreover,

by linking apoptosis, MMP activation and matrix degradation we have demonstrated a potential link by which the controlled loss of the fibrogenic cells (myofibroblasts) and matrix degradation occur during wound healing.

5.10.3 TIMP-2 is known to have a dual role in the regulation of MMP-2 activation being required for the local activation of MMP-2 by MT1 MMP at the cell surface by forming a tri-molecular complex. TIMP-2 has also been shown to complex with proMMP-2 and prevent its activation (Kinoshita et al. 1998). TIMP-1 and TIMP-2 are able to inhibit the activity of active MMP-2 (Brew et al. 2000). TIMP-1, TIMP-2 and a synthetic selective inhibitor of MMP-2 also promote survival of HSC by inhibiting apoptosis.

5.10.4 HSC express a variety of MMPs during activation, but MMP-2 and MMP-14 are persistently expressed in the activated phenotype in vivo and in vitro. For HSC cultured on plastic there is net matrix accumulation as TIMPs are produced preventing matrix degradation. Previous work has demonstrated that TIMP-1 is highly effective at inhibiting active MMP-2. Indeed, dissociation of TIMP-1 from MMP-2 in tissue culture results in approximately a twenty fold increase in gelatinase proteolytic activity (Iredale et al. 1992). MMP-2 activation has recently been demonstrated to occur in association with human HSC apoptosis induced by exposure to cytochalasin D or C(2)-ceramide (Preaux et al. 2002). This has highlighted that MMP-2 may have an important role facilitating HSC apoptosis. As a feature of the transdifferentiation from quiescent stellate cells to the myofibroblast-like HSC, activated HSC increase their synthesis of proMMP-2 (Benyon et al. 1995), TIMP-1 and TIMP-2 (Iredale et al. 1992). The studies so far described in this thesis have demonstrated that MMP inhibition via TIMP-1, TIMP-2 or a synthetic selective inhibitor of MMP-2 can reduce apoptosis of HSC. In contrast, inhibiting TIMP-1 or TIMP-2 with neutralsing antibodies promotes HSC apoptosis confirming that activated HSC produce TIMPs and prevent apoptosis. Given that the balance of TIMP and MMP activity determines HSC fate in this in vitro model, I have studied the effect of altering cell matrix interactions on HSC apoptosis using a mutant type I collagen that is resistant to degradation by MMPs (Krane et al. 1996). This shows that active MMP-2 is capable of promoting apoptosis of human HSC when plated onto plastic or normal type I collagen. Using a mutated type I collagen that is resistant to degradation by MMP-2, further experiments show that human HSC plated onto this substratum is more resistant to apoptosis induced by active MMP-2 than cells plated onto

the wild type, type I collagen. The r/r collagen reduced apoptosis induced by active MMP-2 by approximately 31% (12.5% to 8.7% apoptotic index) compared to wild type collagen. This suggests that protection of intact collagen promotes HSC survival.

5.10.5 This work has important clinical implications for the rational design for new anti fibrotic therapies and suggest that activation of latent proMMP-2 in the fibrotic liver may be a potential method of either promoting or accelerating resolution of liver fibrosis in humans. There have been a number of reports of therapeutic intervention of experimental liver fibrosis that have been reverted by gene therapy delivery of various parts of the matrix proteolysis cascade or their regulators, for example urokinase-type plasminogen activator which initiates the matrix proteolytic cascade (Salgado et al. 2000). Gliotoxin has been shown to promote apoptosis of HSC and has been shown to promote recovery of experimental liver fibrosis in rats (Wright et al. 2001). The data I have described in this chapter show that gliotoxin induced apoptosis of HSC is associated with activation of MMP-2. This further emphasises the concept that promoting apoptosis of HSC in liver fibrosis creates the situation where resolution of liver fibrosis is facilitated. Not only does apoptosis of HSC remove the cells that are responsible for synthesis of excessive collagen and TIMPs, but also during apoptosis of HSC there is activation of latent MMP-2 further aiding degradation of excess matrix proteins.

5.10.6 These further experiments were a natural extension of my previous work by defining a candidate MMP (MMP-2) and suggesting a mechanism whereby the MMP facilitates apoptosis (type I collagen degradation). An interesting observation regarding the experiments with the mutant r/r collagen (Figure 5.7) is that the human HSC plated on the mutant collagen were not completely protected from the pro apoptotic effect of active MMP-2 compared to those plated onto wild type collagen. This suggests that there are other active MMP-2 substrates that impact on human HSC survival. With this in mind, the following chapter examines another potential MMP-2 substrate, namely N-cadherin which mediates cell-cell contacts. In conclusion, the balance of active MMP-2 and TIMPs appear to mediate apoptosis of human HSC at least in part by regulating cell matrix interaction with type I collagen.

Chapter 6

6. Studies of the role of N-Cadherin in activated hepatic stellate cell survival

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6.1 Introduction

6.1.1 The previous work in this thesis has demonstrated firstly that TIMP-1 and TIMP-2 are able to reduce apoptosis of rat and human activated HSC induced by a variety of stimuli. Secondly, TIMP-1 reduces apoptosis in HSC via MMP inhibition. Thirdly, the work has also demonstrated, through a number of complementary experiments, that MMP-2 is a good candidate MMP to play a role in the proteolysis that occurs pericellularly during apoptosis. HSC undergo apoptosis in response to a number of stimuli including serum deprivation, cycloheximide exposure, gliotoxin exposure and nerve growth factor exposure. The first morphologic changes observed after such stimuli are cell retraction and membrane blebbing with subsequent loss of cell-cell and cell-matrix contacts resulting in cell detachment. These observations suggest that cytoskeletal disruption occurs with eventual loss of cell-cell contacts during apoptosis. The work described in the last chapter described how collagen type I degradation could mediate HSC apoptosis induced by active MMP-2. In the following chapter cell to cell interaction is studied in particular focusing on N-cadherin.

6.1.2 Following liver injury resident quiescent stellate cells undergo a phenotypic transformation to the myofibroblast-like activated hepatic stellate cell. The cellular functions change dramatically from one concerned with the storage of vitamin A to a highly active phenotype with multiple new cell behaviours. These include increased proliferation, acquisition of contractile properties, fibrogenesis, matrix degradation, cell migration and chemotaxis of inflammatory cells (Friedman 2000). During spontaneous resolution of experimental liver fibrosis there is loss of the myofibroblast-like cells by apoptosis which correlates with a decrease in TIMP-1 and an increase in matrix degrading metalloproteinase activity (Iredale et al. 1998). This forms a theme that has been observed in a number of pathological situations including resolution of liver fibrosis, resolution of renal glomerulosclerosis and wound healing in the skin (Mizuno-Horikawa et al. 2001; Clark 1993).

6.1.3 The role of TIMP-1 in liver fibrosis has previously been studied in some depth. TIMP-1 promotes accumulation of excess fibrillar collagen by preventing its degradation by directly inhibiting matrix degrading MMPs (Arthur et al. 1989; Iredale et al. 1992), furthermore

studies described in chapters 3 and 4 has described how TIMP-1 prevents apoptosis of the activated HSC via inhibition of MMPs. As a characteristic of the phenotypic change to the myofibroblast like cell, activated HSC synthesize significant quantities of MMP-2 (gelatinase A). A recent report has highlighted that MMP-2 is activated in association with apoptosis of HSC (Preaux et al. 2002), furthermore, the MMP-2 promoter has been shown to contain a functional p53 recognition motif (Bian and Sun 1997) implicating MMP-2 as a key pericellular protease involved in cellular apoptosis. The current model of HSC survival is that in liver fibrosis, the default position for the activated HSC is apoptosis unless the cells are exposed to survival factors (Iredale et al. 1998). Given the dynamic changes in MMP/TIMP-1 balance and HSC population during spontaneous resolution of liver fibrosis and the knowledge that cadherins have been shown to be a substrate for MMPs in some cell types (Herren et al. 1998; Steinhusen et al. 2001), it is appropriate to further investigate the role of cell-cell contacts via N-cadherin and MMP/TIMP-1 balance mediating cell survival.

6.1.4 Cadherins form a diverse family of cell surface molecules and appear to perform a vast array of functions including intercellular recognition, cell adhesion, cytoskeletal organisation, signal transduction, and control of growth (Angst et al. 2001). E-Cadherin and N-Cadherin have been best characterised and studied. They are transmembrane glycoproteins each consisting of five cadherin domains (extracellular domains 1-5 or ECD1-5). Their intracellular regions interact with cytoplasmic proteins β-catenin, plakoglobin, α-catenin and the actin filaments of the cytoskeleton (Angst et al. 2001; Yap et al. 1997a). Beta catenin also binds to the transcription factor lymphoid enhancer-binding factor-1 (LEF-1) providing a mechanism for the transmission of signals from cell adhesion events or the wnt/wingless pathway to the nucleus (Behrens et al. 1996).

6.1.5 Cadherins interact at the cell surface via their first extracellular domains (ECD1) forming homophilic dimers either in trans configuration with other cells or in cis formation with cadherins on the same cell (Yap et al. 1997b). N-Cadherin has also been shown to interact with the fibroblast growth factor receptor via its ECD4 domain (Williams et al. 2001). During apoptosis there is evidence that there is a role for caspases and metalloproteinases in the degradation of β-catenin and VE-Cadherin respectively (Herren et al. 1998). Similarly, E-Cadherin has been shown to be cleaved after induction of apoptosis (Steinhusen et al 2001). by metalloproteinases. Also MMP inhibitors have been shown to increase fibroblast adhesion

through stabilisation of focal adhesion contacts and increase in cadherin function (Ho et al. 2001). TIMP-1 has been demonstrated to inhibit apoptosis of HSC via mechanisms involving inhibition of MMPs (see chapters 3 and 4) highlighting that some MMP substrates can mediate survival of HSC. This has been demonstrated for type I collagen in particular (Issa et al. 2003). Recently apoptosis of HSC has been shown to be associated with activation of MMP-2 (Preaux et al. 2002).

6.1.6 Given the dramatic change in cell function that occurs with HSC activation, it seems likely that associated with these changes are changes in the pattern of cadherin expression of HSC. To some extent cadherins appear to play a role in mediating cell survival in other cell types as blockade of N-cadherin binding with antibodies directed against the extracellular domain of N-cadherin promotes apoptosis of melanoma cells (Li et al. 2001). The peptide sequence HAVDI has been shown to disrupt N-cadherin homodimers by binding to its extracellular domain-1 (Williams et al. 2002) and is thus a specific way of addressing the role N-cadherin homodimerisation in HSC survival.

6.1.7 Study of the role of N-cadherin in activated HSC survival or apoptosis is therefore justified as it is a plausible MMP substrate that might mediate HSC survival. In the following series of experiments I have addressed the hypothesis that N-cadherin is expressed in HSC and promotes their survival. Previous observations that TIMP-1 inhibits apoptosis of HSC via mechanisms involving MMP inhibition has led to further experiments examining the fate of cellular N-cadherin during HSC apoptosis and the effect of altered TIMP/ MMP balance. This chapter describes a series of experiments that demonstrate that following activation of HSC there is up regulation of N-cadherin and that this appears to promote the survival of the HSC. A characteristic feature of HSC activation is the up regulation of synthesis and secretion of progelatinase A or MMP-2 (Benyon et al. 1999). Thus the activated HSC is an ideal choice of material to study the potential role of MMP-2 mediating apoptosis.

6.2 N-Cadherin expression increases with activation of HSC

During activation of HSC there is trans-differentiation to a myofibroblast-like phenotype. Comparison of protein extracts from human HSC extracted after 1 through to 9 days of culture on plastic found that increasing N-Cadherin expression was associated with the process of HSC activation (Figure 6.1).

FIGURE 6.1. EXPRESSION OF N-CADHERIN INCREASES WITH ACTIVATION OF PRIMARY HUMAN HSC CULTURED ON PLASTIC.

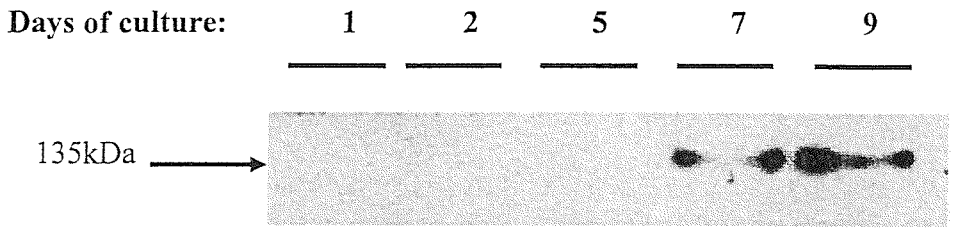


Figure 6.1. Freshly extracted human stellate cells were cultured on plastic as described in the methods and materials. At set time points the cultured cells were harvested and 10μg of the protein extract was separated by SDS PAGE and then blotted and probed for N-cadherin using clone 3B9 antibody. A clear immunoreactive band of appropriate size was detected after 7-9 days in culture.

6.3 Blockade of N-Cadherin binding by blocking antibody promotes apoptosis of HSC

To determine if N-Cadherin had a role in controlling HSC cell survival binding was disrupted by an azide free N-cadherin blocking antibody (Clone GC-4) to an extracellular epitope on N-Cadherin. Blocking with 40μg/ml of antibody was compared to non immune IgG control at the same concentration. In serum free conditions there was a significant increase in apoptosis observed by the acridine orange technique (Figure 6.2). This change was further enhanced in cells that were promoted into apoptosis by co incubation with cycloheximide showing a 7.2% increase in apoptosis compared to non immune IgG control. This suggested that one of the normal functional roles of N-Cadherin in activated HSC is to promote survival. (Figure 6.2).

FIGURE 6.2. BLOCKADE OF N-CADHERIN BINDING WITH ANTI N-CADHERIN ANTIBODY GC-4 TO THE EXTRACELLULAR REGION OF N-CADHERIN PROMOTES APOPTOSIS OF HSC.

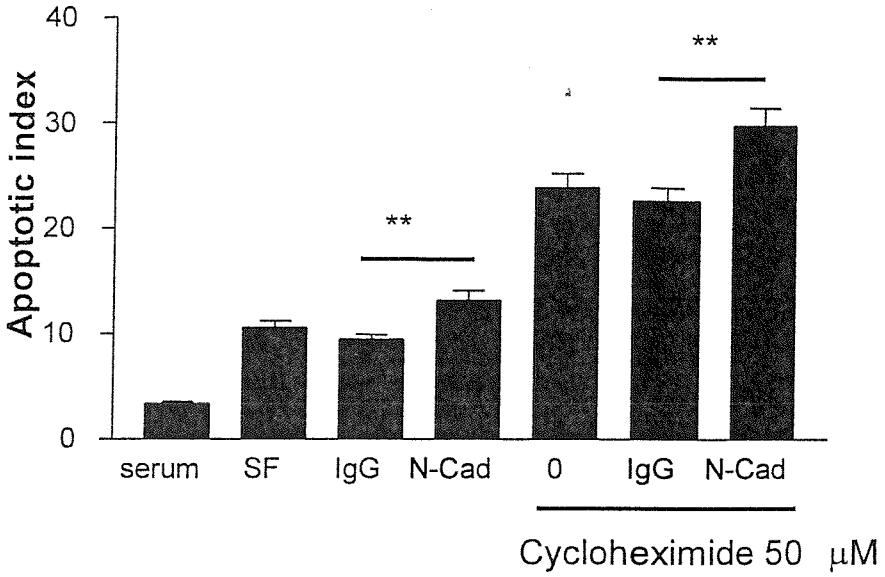


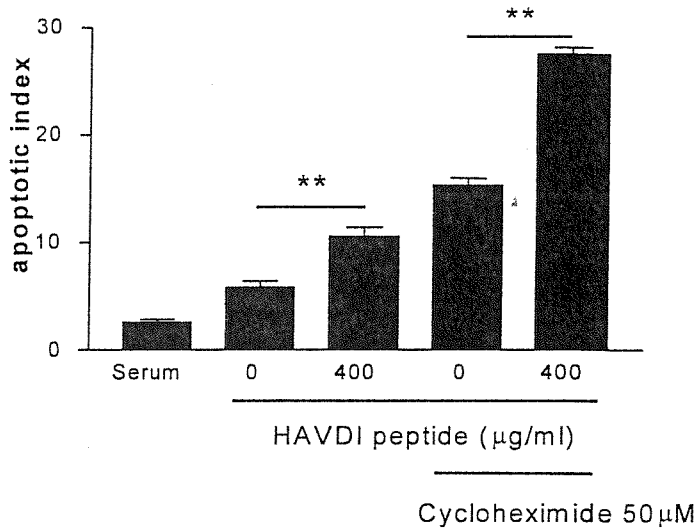
Figure 6.2 Apoptotic indices in human HSC show that the N-cadherin blocking antibody has a modest but significant pro apoptotic effect compared to non immune IgG at the same concentration. Apoptosis was quantified by acridine orange staining and cell counting. (Data expressed are mean ± SEM of apoptotic index. **, $p<0.005$ by Student's t- test, n=5).

6.4 Blockade of N-Cadherin homodimers by HAVDI blocking peptide promotes apoptosis of HSC

To verify the effects of the blocking antibody, the specific peptide antagonist of N-cadherin dimerisation HAVDI, was used to further examine the effect of disrupting N-cadherin homodimerisation on HSC survival. Cultured HSC that had been exposed to the HAVDI peptide for four hours had significantly increased apoptosis compared to cells left in serum free conditions alone (Figure 6.3a). This effect was verified by parallel experiments measuring caspase-3 activity (Figure 6.3b).

FIGURE 6.3A & 6.3B. EFFECT OF N-CADHERIN BLOCKING PEPTIDE HAVDI ON HUMAN HSC APOPTOSIS.

A



B

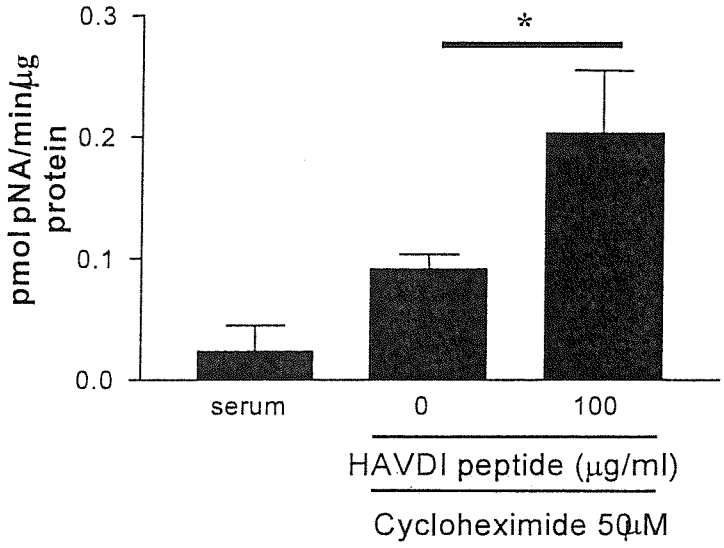


Figure 6.3a & b. A custom peptide "HAVDI" was also used as an alternative means to block N-cadherin homophilic binding. A: The peptide significantly promoted apoptosis of human HSC that were exposed to 400μg/ml of the peptide for four hours compared to cells in serum free media. Apoptosis was quantified by acridine orange staining and cell counting. Data are mean and SEM apoptotic index and are representative of three separate experiments. **$p<0.005$ by Student's t test. B: To verify the pro apoptotic effect of the HAVDI peptide observed by the acridine orange technique, parallel experiments were undertaken and apoptosis was quantified by caspase-3 activity assay. *$p<0.05$ by Student's t-test, n=1.

6.5 N-Cadherin is degraded during HSC apoptosis

Following apoptosis induced by cycloheximide or gliotoxin exposure there was evidence that apoptosis of HSC was associated with N-Cadherin degradation into smaller fragments when assessed by western blotting with antibody clone 3B9 (intracellular epitope) (Figure 6.4). Using an antibody clone GC-4 that recognises the extracellular domain of N-cadherin western analysis of conditioned media from apoptotic cells contained a 70kDa fragment (Figure 6.5).

FIGURE 6.4. WESTERN BLOTTING FOR N-CADHERIN SHOWING EVIDENCE OF DEGRADATION FOLLOWING INDUCTION OF APOPTOSIS BY GLIOTOXIN

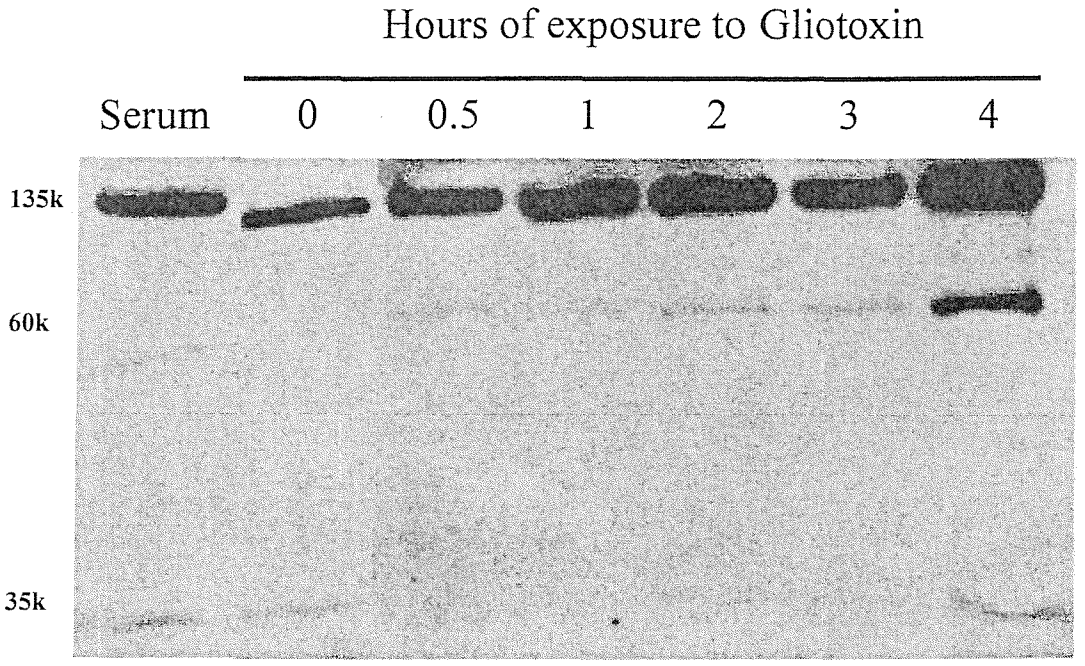


Figure 6.4. Cultured rat HSC were exposed to gliotoxin which is known to be a potent inducer of HSC apoptosis. Cell extracts were made over a time course from 0 to 4 hours exposure to 1.5μM gliotoxin in serum free conditions. Western blotting was undertaken for N-Cadherin using an antibody with an intracellular epitope (Clone 3B9) that did not cross-react with other Cadherin subtypes. Associated with HSC apoptosis was the emergence of smaller immuno-reactive bands around 60kDa in size in addition to the intact size 135kDa N-Cadherin immuno-reactive band. The blot shown is representative of four similar blots with rat or human HSC.

FIGURE 6.5 SUPERNATANT WESTERN BLOT DEMONSTRATING A CLEAVED N-CADHERIN FRAGMENT.

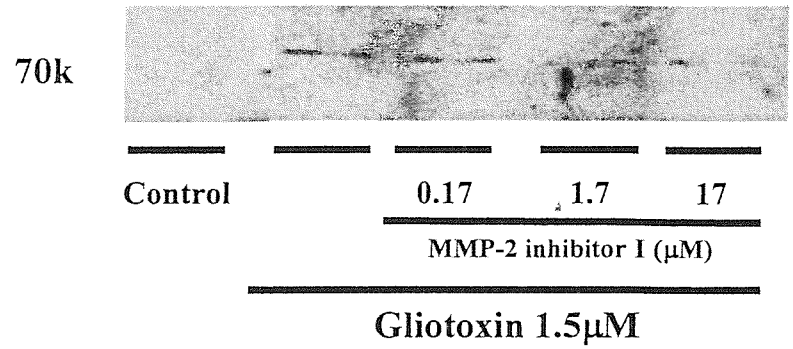


Figure 6.5. To demonstrate cleavage of N-cadherin further, conditioned media was taken from human HSC before and after induction of apoptosis by gliotoxin with and without MMP-2 inhibitor I. The antibody used in the western blot was clone GC-4 which recognises an extracellular epitope on N-cadherin. Exposure to the proapoptotic stimulus gliotoxin yielded a 70k band from conditioned media of human HSC. Conditioned media from human HSC cultured in serum free media from four hours contained no detectable fragment. Parallel experiments with MMP-2 inhibitor I appeared to decrease the quantity of fragment in conditioned media.

6.6 N-Cadherin cleavage during HSC apoptosis is inhibited by TIMP-1 via MMP inhibition

To determine if MMPs were responsible for the degradation of N-Cadherin during HSC apoptosis, parallel experiments were undertaken using MMP inhibitors including TIMP-1 and a mutated non functional T2G N-TIMP-1. Cleavage of N-cadherin was assessed by western blotting using the clone 3B9 antibody that recognises the intracellular end of N-cadherin. While cells in serum demonstrated mostly intact 135kDa N-Cadherin, populations of HSC containing greater numbers of apoptotic cells showed evidence of N-cadherin degradation into fragments ranging in size from 20-100kDa (Figure 6.6). Co-incubation of HSC with TIMP-1 after induction of apoptosis by cycloheximide resulted in reduced fragmentation especially to fragments less than 60kDa in size while a non functional mutant T2G N-TIMP-1 had no effect on HSC apoptosis or N-Cadherin degradation (Figure 6.6).

FIGURE 6.6. TIMP-1 REDUCES N-CADHERIN FRAGMENTATION DURING APOPTOSIS OF HSC BUT THE NON FUNCTIONAL T2G MUTANT TIMP-1 HAS NO EFFECT.

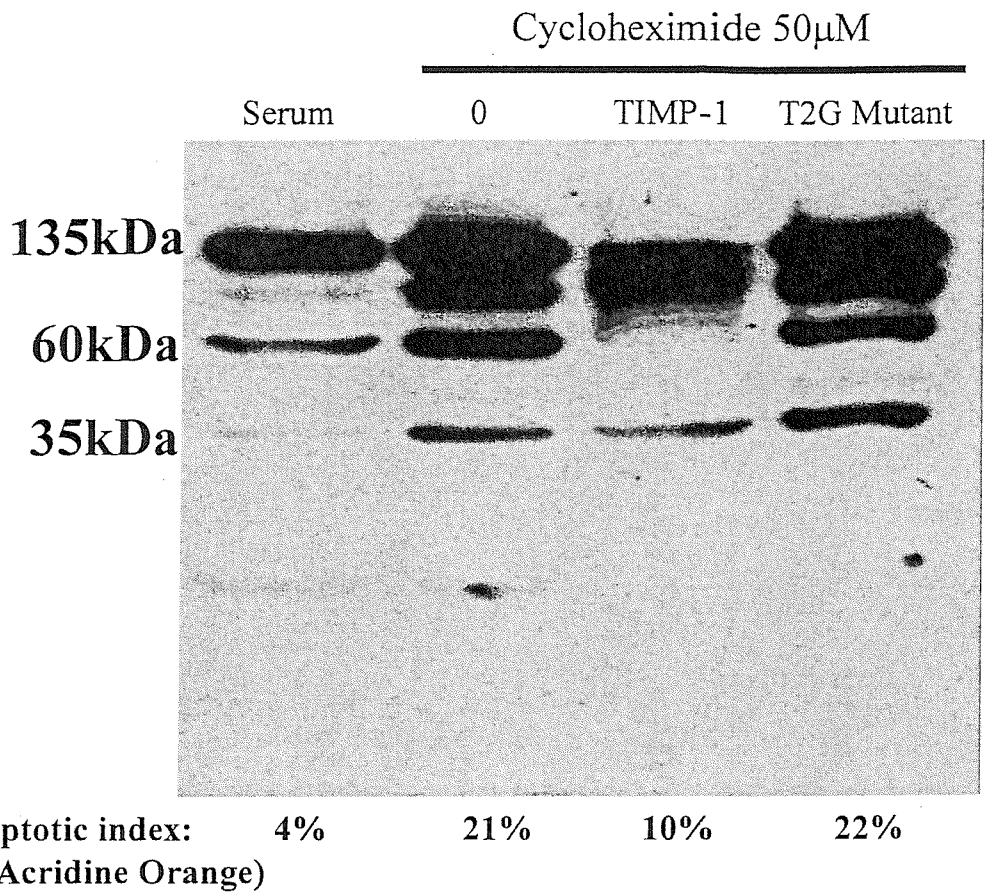


Figure 6.6. To determine the effect of MMP inhibition on fragmentation of N-Cadherin associated with HSC apoptosis cultured HSC were induced into apoptosis by exposure to cycloheximide either alone or with wild type TIMP-1 or a non functional mutant TIMP-1 (T2G mutant). Western blotting for N-Cadherin shows that HSC in serum contain mainly intact 135kDa N-Cadherin. In contrast, HSC induced into apoptosis by cycloheximide contain numerous extra bands ranging in size from 20-100kDa. Co-incubation of HSC with cycloheximide with wild type TIMP-1 reduced the number of extra fragments compared to cycloheximide alone while the T2G non functional mutant TIMP-1 had no effect on N-Cadherin fragmentation. This suggested that MMP proteolytic activity was likely to be involved in the degradation of N-Cadherin.

6.7 Selective inhibition of MMP-2 or MMP-9 prevent cleavage of N-cadherin during apoptosis

Synthetic selective MMP inhibitors were chosen specifically to attempt to selectively inhibit either MMP-1, MMP-2, MMP-3 or MMP-9 as described in the materials and methods. Cultured human HSC were induced into apoptosis to promote cleavage of N-cadherin. Cells grown in serum had intact N-cadherin 135kDa in size. In contrast, cells exposed to cycloheximide showed evidence of N-Cadherin degradation with a strong immuno-reactive band at 60kDa. Cells co-incubated with either a selective MMP-2 inhibitor (M2i) or an inhibitor of MMP-9 (M9i) had intact bands of N-cadherin similar to cells treated with serum alone. In contrast, cells co-incubated with selective inhibitors of MMP-1 or MMP-3 (M1i and M3i respectively) showed decreased amounts of intact N-Cadherin compared to cells treated with serum. This data suggested that MMP-2 played a role in N-cadherin degradation. Also it indicates that neither MMP-1 nor MMP-3 are likely to play a significant role in regulating apoptosis via N-cadherin either because they were simply not present in activated HSC, or because they did not have a role in N-Cadherin degradation in toto (Figure 6.7).

FIGURE 6.7. SCREENING SELECTIVE MMP INHIBITORS.

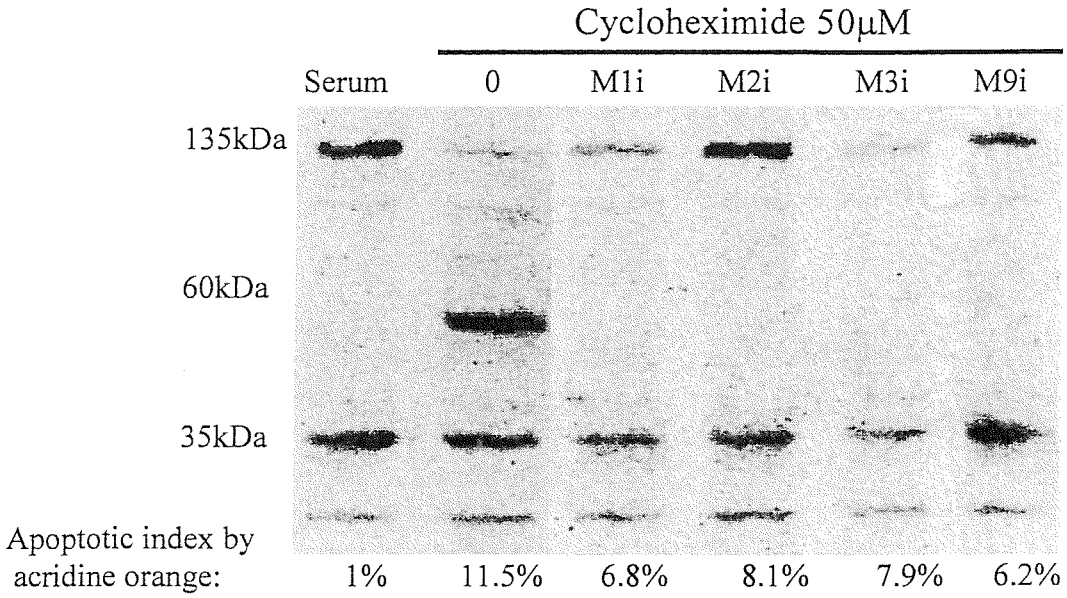


Figure 6.7. Synthetic selective MMP inhibitors were chosen specifically to attempt to selectively inhibit either MMP-1, MMP-2, MMP-3 or MMP-9 (M1i, M2i, M3i & M9i respectively) as described in the materials and methods. Apoptotic indices from parallel experiments are also given to provide a comparison.

6.8 A protein database study predicts MMP-2 as a candidate MMP involved in cleavage of N-Cadherin during apoptosis

Analysis of amino acid sequences of rat and human N-Cadherin demonstrated that the extracellular domain of N-Cadherin contains multiple (approximately 20) potential MMP cleavage sites containing the potential MMP cleavage motif Pxx/Hy (where P is proline, x is any amino acid and Hy is any hydrophobic amino acid. More specific motif searches showed that MMP-2 is a good candidate MMP responsible for N-Cadherin degradation with five potential cleavage sites by amino acid sequence alone. Four out of the five sites found were conserved in rat and human N-cadherin. Active MMP-2 cleavage motifs used were LIxx/Hy, HySx/L and Hxx/Hy (Chen et al. 2002).

FIGURE 6.8. PROTEIN DATABASE STUDY OF MMP-2 CLEAVAGE MOTIFS WITHIN N-CADHERIN.

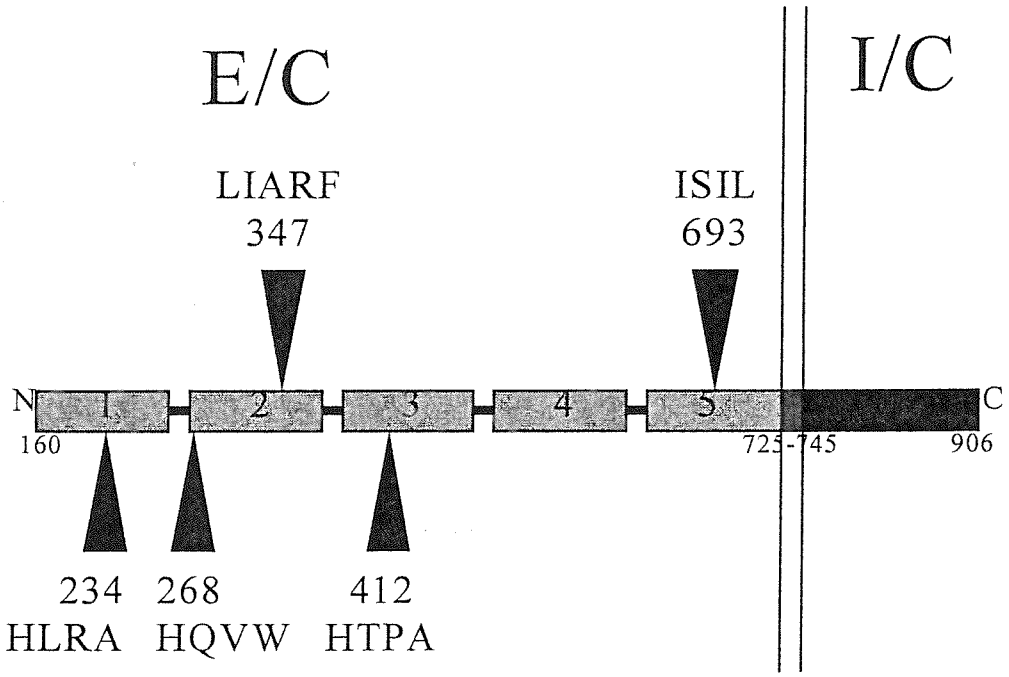


Figure 6.8 Protein data base Swiss Prot was used to access amino acid sequences of human N-cadherin (Accession number P19022). A search of the amino acid sequence using published MMP-2 cleavage motifs showed five potential cleavage sites. The above figure shows each of the five extracellular cadherin domains (1-5), other figures are the amino acid number, location and sequence of the found motif. (E/C extracellular domain, I/C intracellular domain).

6.9 Active MMP-2 promotes N-Cadherin cleavage in cultured HSC

To determine if active MMP-2 promoted N-Cadherin cleavage in cultured human HSC, cultured cells were exposed to active MMP-2 with cycloheximide to promote HSC apoptosis.

After four hours cells were harvested and analysed by western blotting for N-Cadherin. Exposure of HSC to active MMP-2 with cycloheximide promoted apoptosis and parallel experiments demonstrated enhanced fragmentation of N-Cadherin in cells exposed to active MMP-2 (Figure 6.9).

FIGURE 6.9. THE DIRECT EFFECT OF ACTIVE MMP-2 ON CULTURED HSC N-CADHERIN.

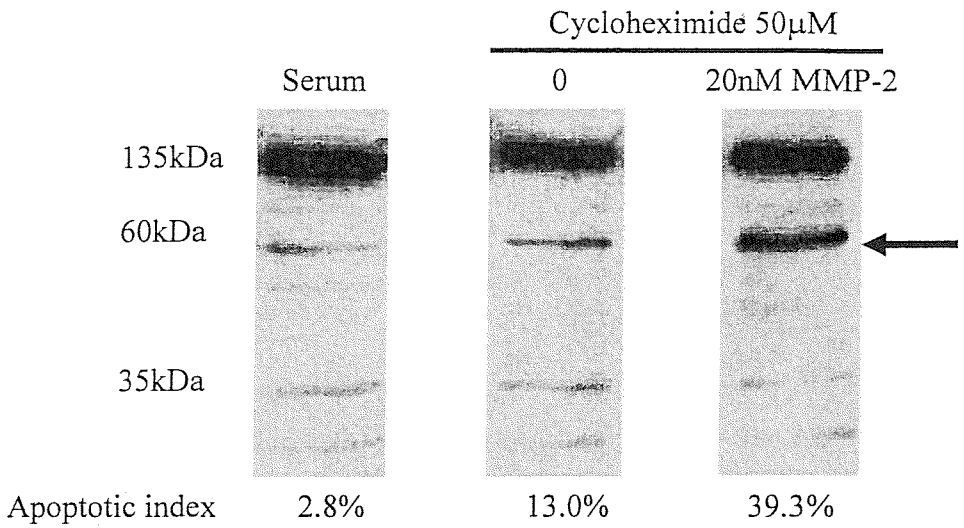


Fig.ure 6.9. To determine the effect of active MMP-2 on cultured HSC N-Cadherin, cultured HSC were exposed to active MMP-2 (20nM) for four hours then cells were harvested and proteins extracted as described in methods and materials. More of the 60kDa fragment was present in extracts of cells treated with active MMP-2 compared to cells treated with cycloheximide alone (see arrow). This suggested that active MMP-2 had a direct effect on N-Cadherin in culture activated human HSC.

6.10 Active MMP-2 cleaves N-Cadherin in vitro

To determine if active MMP-2 directly cleaves N-Cadherin, 10ug of HSC protein extract was exposed to MMP buffer alone or 1ug of active MMP-2. These samples were incubated for 1 hour at 37°C then immediately run on SDS PAGE gel followed by western blotting for N-Cadherin as before. After 1 hour incubation the intact N-cadherin represented by the immunoreactive 135kDa band was completely removed (Figure 6.10). In contrast the beta actin controls from the same blot remained intact. This suggested that active MMP-2 could directly cleave N-Cadherin in vitro.

FIGURE 6.10. ACTIVE MMP-2 DEGRADES N-CADHERIN.

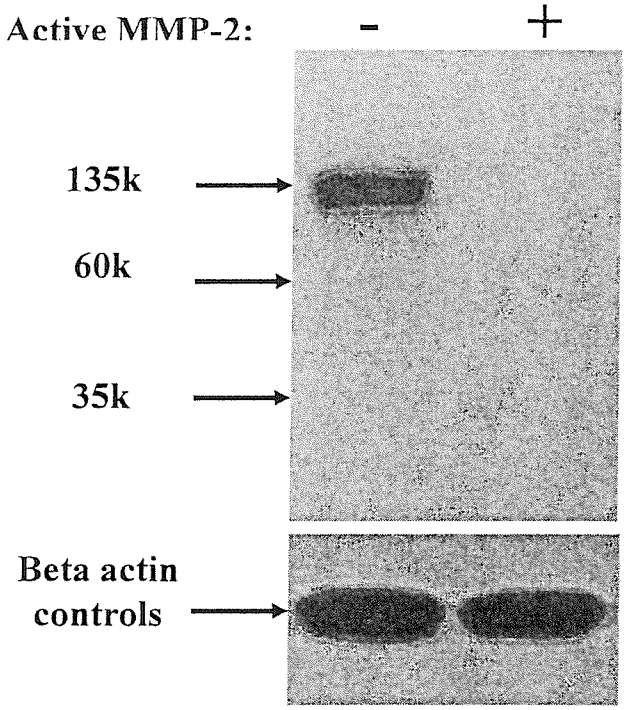


Figure 6.10. To determine if active MMP-2 directly cleaved N-Cadherin 10ug of serum starved HSC protein extract was used a substrate for MMP-2. Protein extract was incubated for 1 hour either with MMP buffer alone (-) or with 1ug of active MMP-2 (+). Samples were then immediately run on SDS PAGE gel and western blot undertaken for N-Cadherin using clone 3B9. Without MMP-2 digestion there is an intact band of N-Cadherin around 135kDa in size, with MMP-2 digestion the 135kDa band has disappeared and only a small 35kDa band remains. The blot was stripped and probed for beta actin as shown to confirm equal protein loading. Data shown are representative of three separate experiments. This suggests strongly that active MMP-2 can degrade N-cadherin from human HSC in vitro.

6.11 Human HSC express N-cadherin at the cell membrane with beta catenin

Staining for N-Cadherin intracellular moeity demonstrated that in human HSC N-Cadherin is located at the cell surface and at the cell surface membrane as would be expected. In addition, parallel staining demonstrated that beta catenin was also localised mainly to the cell membrane (Figure 6.11).

FIGURE 6.11. STAINING FOR N-CADHERIN AND BETA CATENIN IN HUMAN HSC.

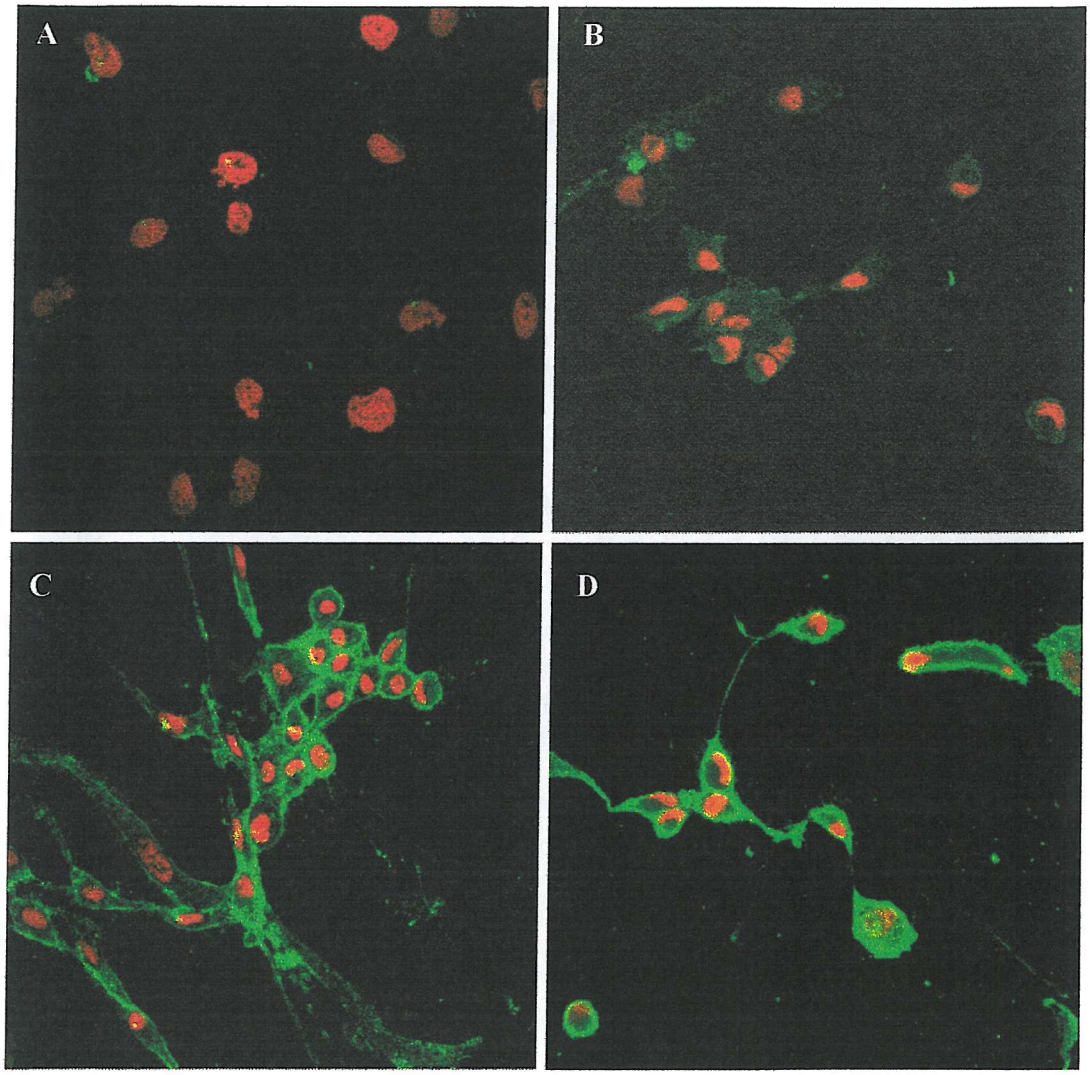


Figure 6.11. Laser confocal imaging Z series of human HSC cultured in serum containing media. A and B are negative controls treated without a primary antibody and stained with 7-actinomycin-D to show nuclei. C: Staining for N-cadherin (intracellular epitope) reveals a membrane location for N-cadherin. D: Staining for beta catenin reveals its location is also closely associated with the cell membrane. (Images are representative of two separate experiments, magnification x40).

6.12. Active MMP-2 affects the cellular distribution of N-cadherin and β-Catenin

Laser confocal microscopy was undertaken to determine if changing the balance of TIMP and MMP-2 effected the distrubution of β-Catenin which is known to be one of the downstream intracellular signalling pathways in which N-Cadherin participates. Parallel experiments staining for β-Catenin were undertaken. Cells cultured in serum free media had N-cadherin and β-Catenin mainly located at the cell membrane (Figure 6.12A & B respectively). After exposure to active MMP-2 for 4 hours N-cadherin and β-Catenin distribution changed to a floccular pattern in the cytosol (Figures 6.12C & D respectively). Exposure of human HSC to 17μM of the MMP-2 inhibitor I maintained the distribution of N-cadherin and β-catenin to the cell membrane (Figures 6.12 E & F respectively).

6.13 The effect of MMP inhibition on distribution of β-catenin during apoptosis

To determine the fate of N-cadherin and beta catenin during human HSC apoptosis, cells were induced into apoptosis by exposure to gliotoxin and then stained for N-cadherin and β-catenin. Exposure to gliotoxin was associated with a marked redistribution of N-cadherin and β-Catenin from the membrane to a cytoplasmic location (Figure 6.13 compare A & B with C & D respectively). Co-incubation with gliotoxin and the MMP-2 inhibitor I maintained the N-cadherin and β-catenin in a cell surface membrane distribution confirming that active MMP-2 is responsible for cleavage of N-cadherin (Figure 6.13 compare C & D with E & F).

6.14 TIMP-1 and TIMP-2 maintain N-cadherin at the cell surface

To further study the anti apoptotic effect of TIMP-1 and TIMP-2, human HSC were exposed to gliotoxin or gliotoxin with TIMP-1 or TIMP-2. Cells were then stained for N-cadherin as described in the methods section. Following exposure to gliotoxin (0.375μM for four hours) staining was dramatically reduced (Figure 6.14C). In contrast cells that were in serum (Figure 6.14B) or cells that were exposed to gliotoxin with MMP-2 inhibitor I, TIMP-1 or TIMP-2 (Figures 6.14D, E & F respectively) appeared have N-cadherin maintained on the cell surface. This provides further mechanism to explain how MMP inhibitors promote survival of HSC.

FIGURE 6.12 LASER CONFOCAL IMAGES OF HUMAN HSC WITH ACTIVE MMP-2 OR MMP-2 INHIBITOR.

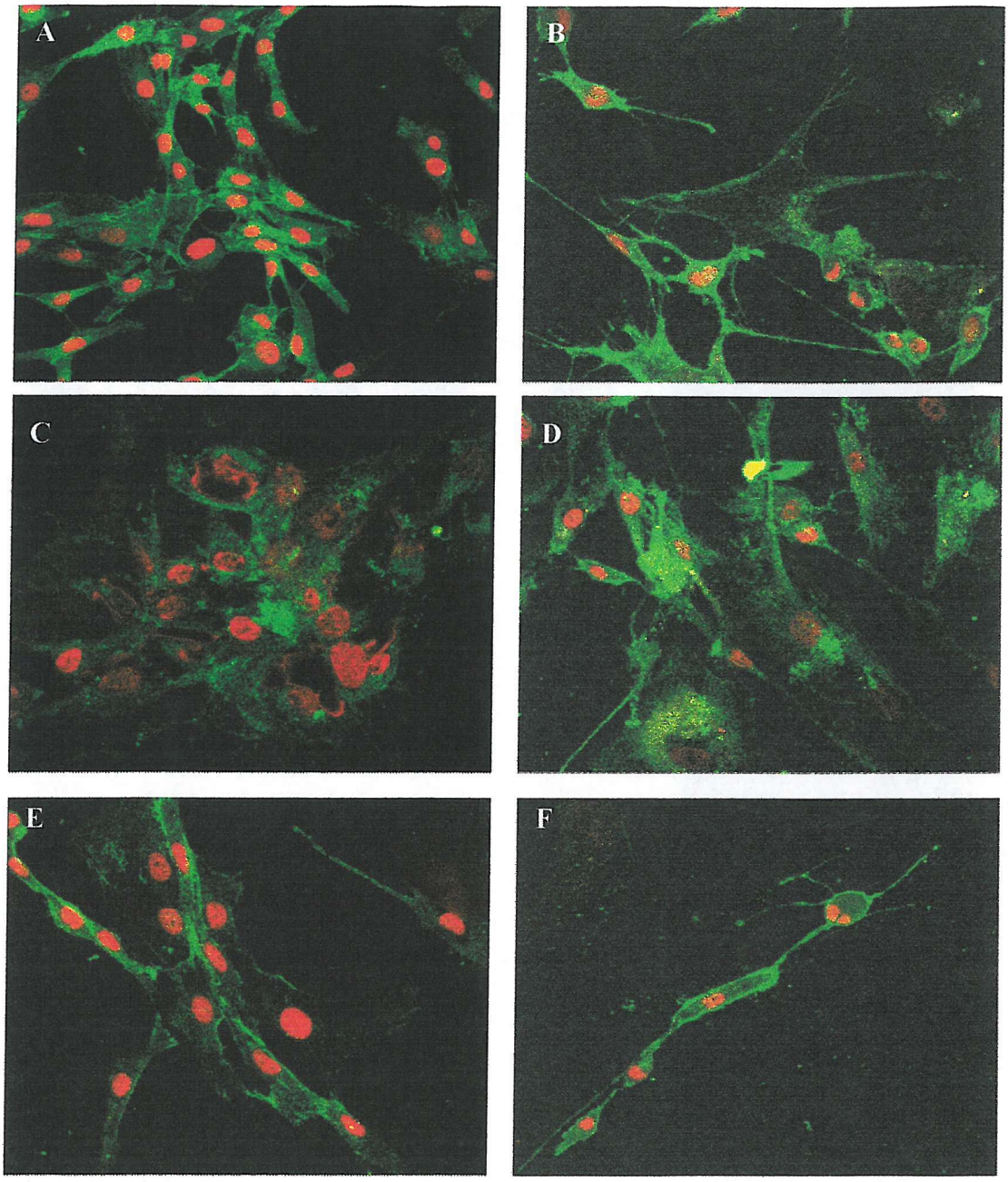


Figure 6.12. Laser confocal imaging was undertaken to examine the effect of excess active MMP-2 versus excessive inhibition of MMP-2 with an inhibitor of MMP-2. A & B: Human HSC in serum free conditions stained for N-cadherin intracellular epitope and beta catenin respectively. C & D: Human HSC exposed to 10nM of active MMP-2 for four hours then stained for N-cadherin and beta catenin respectively. E & F: Human HSC in serum free conditions with the presence of 17μM MMP-2 inhibitor for four hours than stained for N-cadherin and beta catenin respectively (Images are representative of two separate experiments, magnification x40).

FIGURE 6.13. LASER CONFOCAL IMAGES OF HUMAN HSC EXPOSED TO GLIOTOXIN TO INDUCE APOPTOSIS AND THE INFLUENCE OF MMP-2 INHIBITOR I.

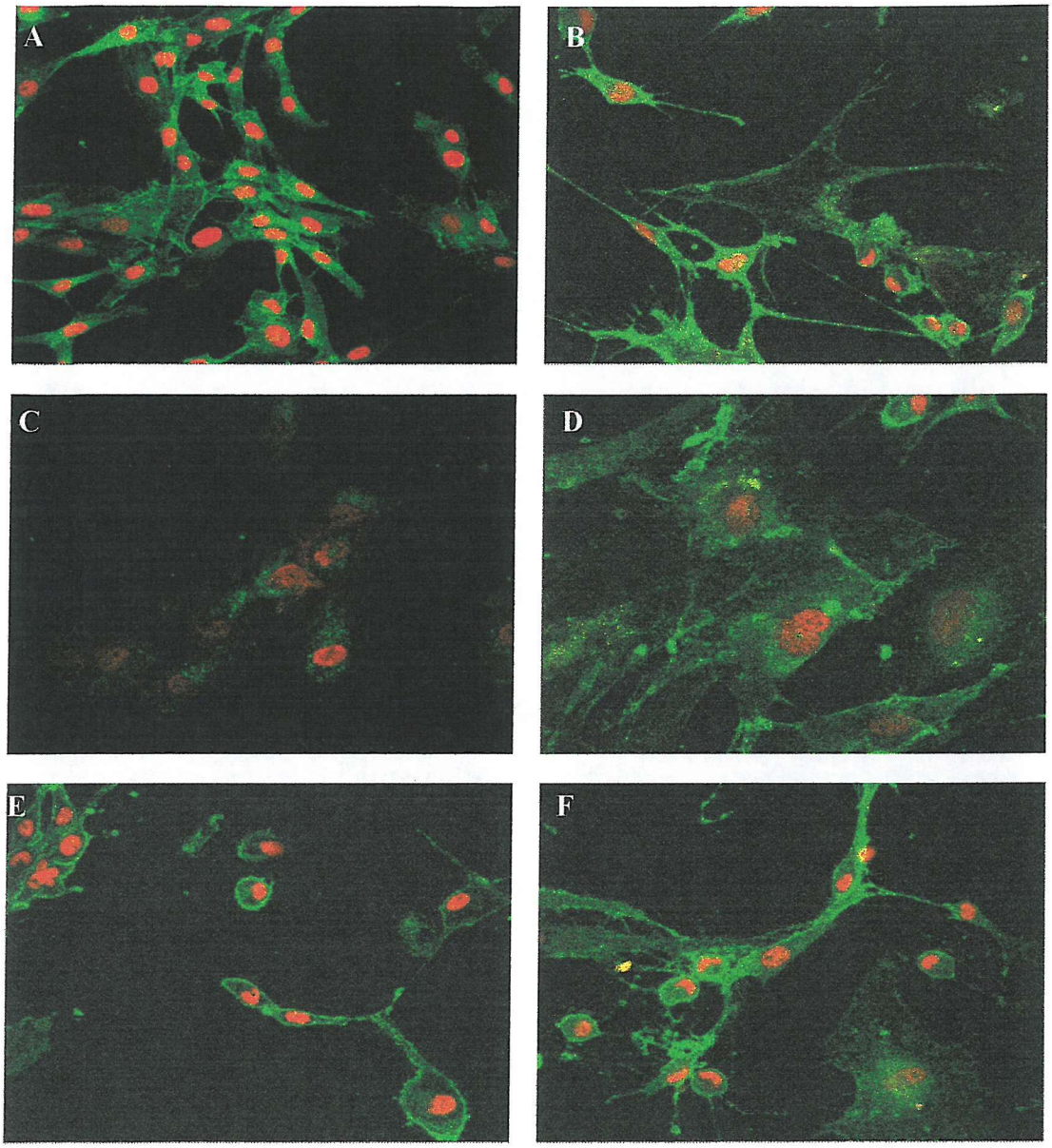


Figure 6.13. Further immunostaining and laser confocal was undertaken to examine the changes in N-cadherin and β-catenin distribution in human HSC that were promoted into apoptosis by exposure to gliotoxin (0.375μM for four hours). A & B: Human HSC in serum free media and stained for N-cadherin and β-catenin respectively show mainly cell membrane distribution. C & D: human HSC exposed to gliotoxin and stained for N-cadherin & β-catenin respectively shows a dramatic change in N-cadherin (compare plate A & C) and β-catenin (compare plates B & D) from membrane location to the cytoplasm. E & F: Human HSC exposed to gliotoxin and 17μM MMP-2 inhibitor I and stained for N-cadherin and β-catenin respectively shows that MMP-2 inhibition maintains the membrane location of N-cadherin and β-catenin (compare plates C & E, and plates D & F). (Images are representative of two separate experiments, magnification x40).

FIGURE 6.14. N-CADHERIN IS PROTECTED BY MMP INHIBITORS.

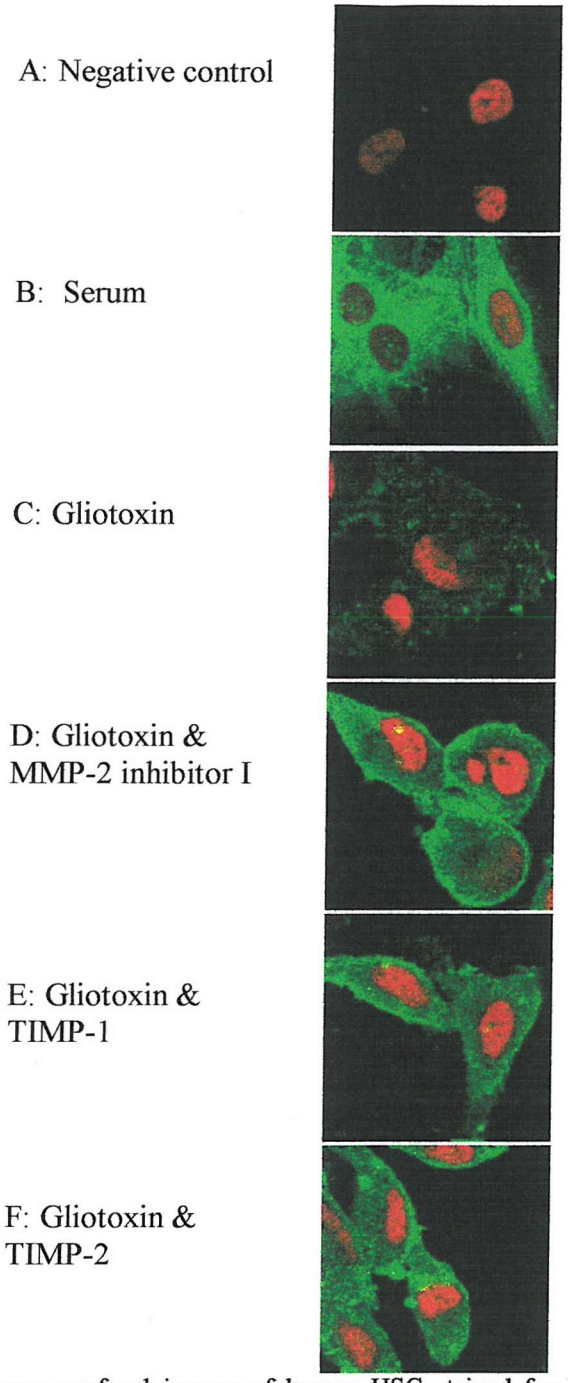


Figure 6.14. Laser confocal images of human HSC stained for N-cadherin intracellular motif. A: Negative control. B: Human HSC in serum. C: Human HSC exposed to gliotoxin (0.375μM) for four hours. D: Human HSC exposed to gliotoxin and MMP-2 inhibitor I (17μM). E: Human HSC exposed to gliotoxin and TIMP-1 (10nM). D: Human HSC exposed to gliotoxin and TIMP-2 (10nM). The images demonstrate that both TIMP-1 and to a lesser extent TIMP-2 facilitate maintenance of N-cadherin at the cell surface. (Images are representative of two separate experiments, magnification x40).

6.15 Summary of the findings from study of the role of N-cadherin on HSC survival

1. Activated human and rat HSC express N-cadherin at their cell surface
2. Blockade of N-cadherin homodimers by antibody or HAVDI peptide promotes apoptosis of activated HSCs
3. Apoptosis of HSC is associated with proteolytic degradation of N-cadherin by MMPs
4. N-cadherin is a substrate for active MMP-2
5. Inhibition of MMPs by TIMP-1, TIMP-2 or MMP-2 inhibitor I maintains N-cadherin and β-catenin at the cell surface even after an apoptotic stimulus by gliotoxin
6. HSC survival is mediated at least in part by the balance of active MMP-2 and TIMPs

6.16 Discussion

6.16.1 The experiments I have described in this chapter clearly demonstrate that human HSC express N-cadherin. Following induction of apoptosis of HSC there is degradation of N-cadherin mediated by MMP-2 with release of N-cadherin fragments from the cells. MMP-2 appears to have a role mediating apoptosis of HSC as inhibitors of MMP-2 prevent N-cadherin degradation and apoptosis of HSC. Following liver injury the stellate cells undergo a phenotypic change to myofibroblast-like cells. With this change in phenotype are changes in the proteins that the cells express. Activated HSC have been known to synthesize proMMP-2 during liver injury. During resolution of liver injury reports have demonstrated that MMP-2 is activated and participates in matrix degrading activity (Salgado et al. 2000). The molecular process behind spontaneous resolution of liver fibrosis is probably a highly organised cascade of activation of many different MMPs of which MMP-2 is merely a single example and has matured to become the final focus in this thesis (Friedman 2000).

6.16.2 A theme that has emerged with resolution of fibrosis is that there is loss of activated HSC by apoptosis with associated evidence of MMP activity. The work described in this chapter has shown that N-cadherin provides a survival stimulus for activated HSC as blockade of N-cadherin binding by antibody or blocking peptide promotes apoptosis. During apoptosis of HSC there is degradation of N-cadherin into smaller fragments. Proteases involved are likely to be MMPs because while TIMP-1 reduced N-cadherin fragmentation, a mutant TIMP-1 without inhibitory activity could not prevent fragmentation. It is self evident that in primary cultures of activated HSC for an MMP or MMPs to be involved in apoptosis of HSC they will need to be present. MMP-2 satisfies this criterion as previous reports have

shown that activated human HSC synthesize MMP-2 furthermore data described in the last chapter has shown activation of MMP-2 with human HSC apoptosis. This observation has been shown by another group in rat HSC (Preaux et al. 2002).

6.16.3 Experiments using the selective inhibitor of endogenous MMP-2 prevented cleavage of N-cadherin in HSC in cells promoted into apoptosis by cycloheximide further implicating MMP-2 as an MMP capable of degrading N-Cadherin. Bioinformatic protein database studies also supported the idea that active MMP-2 is capable of degrading N-Cadherin. Active MMP-2 cleaved N-Cadherin in protein extracts taken from HSC in vitro and exposure to active MMP-2 was associated with a change in the distribution of β-catenin in cultured HSC while TIMPs maintained N-cadherin and β-catenin at the cell surface even after a potent pro apoptotic stimulation with gliotoxin.

6.16.4 Overall, these data indicate that MMP-2 has a role mediating apoptosis of HSC by degrading N-Cadherin. This hypothesis would fit with other observations from experimental models of spontaneous resolution of liver fibrosis that demonstrate activation of MMP-2. A potential criticism of this work is that previous reports have suggest that MMP-2 acts as a mitogen in activated HSC (Benyon et al. 1999). It is however entirely possible that MMP-2 has different roles in liver fibrogenesis compared to fibrosis recovery. Indeed, the cellular response to MMPs has been shown to some extent to be context dependent as data presented in the previous chapter showed that at low concentrations active MMP-2 acted as a mitogen but when cells were exposed to other mitogens for example PDGF or IGF-1 the mitogenic effect of MMP-2 was less evident. MMP-2 at a higher concentration promoted apoptosis. These data point for a potential role for MMP-2 in providing an extra-cellular signal that mediates or facilitates HSC apoptosis.

6.16.5 From a functional perspective MMPs are a major group of enzymes that participate in ECM turnover. There is however an expansion of work demonstrating that the substrate reportoire of MMPs is wide and include non matrix proteins including cell-cell surface molecules (Herren et al. 1998). The interaction of cells with ECM is crucial for the normal development and function of multicellular organisms. Equally, cell-cell interactions and their down stream pathways play a key role in development. β-catenin knockout mice demonstrate this elegantly as mice deficient in β-catenin fail to develop mesoderm or head structures

(Huelsken et al. 2000). One possible generic function for MMPs may be that of a facilitative role in cell biology that involves alterations in pericellular proteins and the cytoskeleton in general. This would include cytoskeletal changes observed in cell proliferation, apoptosis and cell migration or metastasis. Overall this would render the biological response of a given cell to MMP activity highly dependent on the cell context. In the case of the activated HSC, there is evidence that the default position for this cell type is apoptosis which is otherwise forestalled by the presence of other stimuli. TIMP-1 and TIMP-2 are examples of stimuli that prevent apoptosis of HSC by preventing degradation of N-cadherin.

6.16.6 During spontaneous recovery from liver fibrosis there is a dramatic fall in TIMP-1 expression in whole liver allowing free active MMP activity thereby removing many survival signals that the HSC are exposed to (Iredale et al. 1998). With the fall in TIMP-1 there is an increase in collagenolytic activity by MMPs leading to matrix degradation. There is degradation of type I collagen which has also been shown to provide a survival signal for activated HSC (Issa et al. 2003). N-Cadherin is involved in cell to cell binding and is also another adaptive protein associated with the activated HSC phenotype providing a survival stimulus that is maintained provided there is inhibition of MMP activity by TIMPs. In conclusion, the balance of TIMP-1 and MMP-2 may determine HSC fate through either stablisation or degradation of N-cadherin respectively.

Chapter 7

7.1. The relevance of the work to our understanding of liver wound healing

7.1.1 In the last four chapters I have described a series of detailed studies into the effect of TIMP-1 and TIMP-2 on hepatic stellate cell survival. Initial data obtained by comparing the 6 and 12 week rat models of liver fibrosis/cirrhosis demonstrated a correlation between spontaneous resolution of fibrosis, loss of HSC, falling TIMP-1 mRNA expression and transient increases in collagenolytic activity in liver homogenates. This correlative data provided a focus that TIMP-1 or TIMP-2 influenced HSC survival. The in vitro studies that followed clearly demonstrated that TIMP-1 and TIMP-2 inhibited HSC apoptosis induced by a variety of stimuli including serum deprivation or exposure to cycloheximide, gliotoxin and nerve growth factor. TIMP-1 and TIMP-2 were also demonstrated to behave as autocrine survival factors for HSC. Mechanistic studies of the anti apoptotic effect of TIMP-1 revealed that it was via inhibition of MMP activity and that apoptosis regulating gene expression was changed after exposure to TIMP-1 relative to the mutant T2G TIMP-1. Investigation of three different active MMPs revealed that to some extent, MMP-2, MMP-3 and MMP-9 were all pro apoptotic.

7.1.2 This prompted the study of active MMP-2 on cultured HSC. MMP-2 was a natural choice because it is well known to be synthesised by active HSC (Benyon et al. 1999). In chapter 5, I described how active MMP-2 promotes or facilitates apoptosis of cultured HSC by contributing to the degradation of type I collagen furthermore the pro apoptotic effect of active MMP-2 could be inhibited by commercial MMP-2 inhibitors, TIMP-1 or TIMP-2. This effect also highlighted that type I collagen was unlikely to be the only active MMP-2 substrate that mediated HSC survival as cells plated onto mutant r/r collagen were not completely resistant to the pro apoptotic effect of active MMP-2. This led to the studies of cell-cell interaction via N-cadherin in promoting human HSC survival. In chapter 6, I described how N-cadherin expression increases during the process of stellate cell activation and that N-cadherin is a substrate for active MMP-2. Blockade of N-cadherin binding by blocking antibody or HAVDI peptide promoted HSC into apoptosis and suggested that N-cadherin binding promotes HSC survival. Finally, I demonstrated that N-cadherin is degraded by active MMP-2 in HSC following induction of their apoptosis.

7.1.3 Together, this data therefore supports the hypothesis that TIMPs promote the survival of HSC via inhibition of MMP activity. This conclusion is born out by the later complementary studies demonstrating an MMP and two of its substrates that have influence over HSC apoptosis. I therefore propose a novel model for the role of TIMP-1 and MMP-2 in spontaneous recovery from liver fibrosis (Figure 7.1). In liver fibrosis, HSC survival is maintained by survival stimuli in particular cell matrix interactions via integrins to type I collagen, and cell to cell interactions via cadherins. In liver fibrosis, the net MMP activity is held in check by TIMP-1 or TIMP-2 thereby promoting matrix accumulation. After cessation of liver injury, there is a natural tendency over time for wound resolution to occur. During spontaneous resolution of fibrosis the net MMP activity increases overcoming inhibition by TIMPs. A component of this increase in MMP activity is the activation of latent MMP-2 that occurs with apoptosis of HSC. The active MMP-2 acts locally on pericellular substrates for example type I collagen and N-cadherin removing survival stimuli for other HSC. Apoptosis of HSC therefore follows by default and with this there is loss of the cells responsible for type I collagen synthesis and the TIMPs that prevent matrix degradation. In areas of fibrotic bands populated by HSC, the apoptosis of HSC and accompanying local activation of latent MMP-2 allows the discrete localised degradation of the fibrotic bands that is observed in the 6 week carbon tetrachloride model. As the population of HSC decreases to low numbers the net MMP activity also returns to normal.

7.1.4 In support of this idea is the observed magnitude of change in collagenotytic activity from the period of recovery of liver fibrosis following 6 weeks of injury with carbon tetrachloride. The collagenolytic activity in liver homogenates were 70% at peak fibrosis, 147% at day 5 and 107% at day 15 of spontaneous recovery (Data expressed relative to that for normal liver, see section AII.5). So between day 0 and day 5 there is an approximately two fold increase in net collagenolytic activity. The increase in MMP-2 activity associated with apoptosis of HSC in my in vitro experiments were of a similar order of magnitiude (approximately 2-3 fold). It is therefore plausible that the activation of MMP-2 observed with apoptosis of HSC contributes at least in part to that observed in the in vivo model.

FIGURE 7.1. THE MODEL OF TIMP-1 PERICELLULAR EFFECTS IN LIVER FIBROSIS VERSUS RESOLUTION OF FIBROSIS.

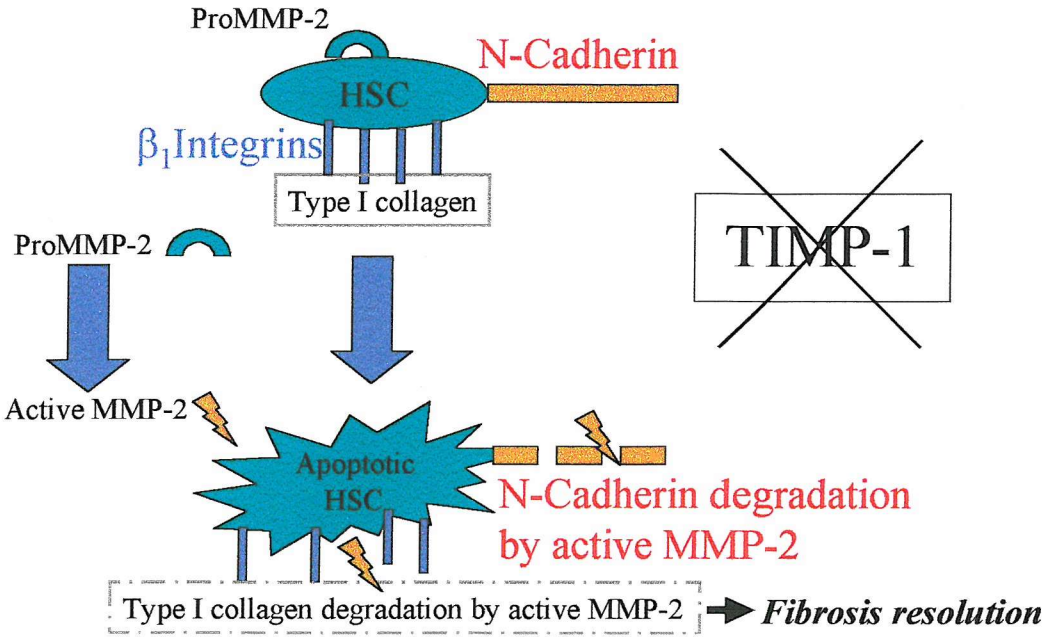


Figure 7.1. The model of TIMP-1 pericellular effects proposes that in liver fibrosis, HSC survival is maintained by TIMP-1 mediated protection from MMP degradation of survival stimuli provided by type I collagen and N-cadherin. During resolution of liver fibrosis there is activation of MMP-2 which degrades type I collagen and N-cadherin, thereby removing survival stimuli for the HSC which then default to apoptosis. Thus, fibrosis resolution occurs.

7.1.5 Overall, the data presented in this thesis provides support for another hypothesis that the default position for the activated HSC is apoptosis which is otherwise forestalled by survival stimuli. The work has ended focusing on the role of type I collagen and N-cadherin because they represent generic examples of cell-matrix and cell-cell survival stimuli the HSC are likely to experience. It is plausible that there are numerous other survival stimuli which could be regulated by the pericellular balance of TIMPs and MMPs. The work is further supported by the recently published findings of other research groups. In particular, I refer to the observation that during apoptosis of HSC there is increased activation of MMP-2. This was also shown in work by Preaux et al. (2002) where apoptosis was induced by Ceramide or Cytochalasin D. In part of my work gliotoxin was used to induce apoptosis. Direct

measurement of active MMP-2 using the Biotrak activity assay from Amersham demonstrated a 2-3 fold increase in active MMP-2 in supernatants from apoptotic HSC compared to HSC left in serum free media alone after 4-6 hours. This increase in MMP-2 activity following induction of HSC apoptosis links a number of observations from in vivo models. It explains the correlation between falling TIMP-1, apoptotic HSC, transient increase in net collagenolytic activity and loss of fibrotic bands that is observed in the 6 week experimental model of liver fibrosis described in chapter 3. It also provides a mechanism to explain why gliotoxin promotes resolution of experimental liver fibrosis in rats (Wright et al. 2001). Another supporting line of evidence for a role of MMP-2 in apoptosis is the observation that MMP-2 has a unique localising mechanism of activation at the cell surface by MT1 MMP and TIMP-2 (Strongin et al. 1995) and that MMP-2 transciption is regulated in part by p53 (Bian & Sun 1997). Discovering that active MMP-2 promotes apoptosis of HSC and that apoptosis is associated with increased activation of MMP-2 is a paradigm shift in our understanding of the its role in liver fibrosis. Previous reports have shown that MMP-2 promotes proliferation of HSC (Benyon et al. 1999). This observation was confirmed in my own experiments at low concentrations (0.1nM) of active MMP-2. However, in the presence of other growth factors (PDGF or IGF-1), an additional proproliferative effect of active MMP-2 was not observed. Indeed, the biological response of HSC to active MMP-2 proved to be highly context dependent. It is entirely possible that active MMP-2 has different roles in liver fibrogenesis compared to resolution.

7.1.6 Two questions that arise from this model are: what makes up the dominant MMPs involved in spontaneous resolution? Also, what are the earliest factors that promote the injured liver to move from fibrogenesis to spontaneous resolution? As I described in the introdution, the are at least five different described MMPs expressed in liver disease, namely MMP-1, MMP-2, MMP-3, MMP-9 and MT1 MMP. A major difficultly studying MMPs is that their activity is so tightly regulated that in vitro studies represent at best "smoking gun" data. For example, the levels of active MMP-2 that I found in conditioned media from human HSC using the Biotrak activity assay were surprisingly low of the order of 10pM (620pg/ml, see Figure 5.2). It is however entirely possible that the true concentration of active MMP-2 at the cell membrane where activation occurs may be much higher. In support of this is the observation that the concentration of TIMP-1 or TIMP-2 required to reduce apoptosis in vitro was of the order of 5nM some five hundred times higher in concentration.

7.1.7 These studies have addressed a crucial question to our understanding of liver fibrosis: What determines whether a fibrotic liver injury recovers or fails to recover? The starting point for the studies described in this thesis was the observation that in recovery there is a net reduction in activated HSCs and fibrotic matrix, while in progressive fibrosis the activated HSCs and neo-matrix remain. Identification of factors promoting the survival of activated HSC is therefore essential to understanding the pathogenesis of fibrosis. I have demonstrated that TIMP-1 and TIMP-2 are important mediators of HSC survival. Our conclusion that TIMP-1 promotes survival of HSC by inhibiting apoptosis is borne out by recent work that demonstrated significantly increased levels of fibrosis and HSC in transgenic mice that over expressed TIMP-1 compared to wild type mice following liver injury with carbon tetrachloride (Yoshiji et al. 2000). This indicates that TIMP-1 has a biologically significant effect promoting fibrosis in vivo. It is important to remember however there are other important factors that influence resolution of liver injury. The role of hepatocyte regeneration is important and was discussed in the introduction to this thesis in depth and it is timely to remember that activated HSC also interact with many other cell types in the liver including hepatocytes, endothelial cells, Kupffer cells and other inflammatory cells.

7.1.8 An important question that has not been addressed in this work is what is the cellular origin of the MMPs that cause the transient increase in collagenolytic activity observed in livers undergoing spontaneous resolution of liver fibrosis. This MMP activity is likely to be made up of the co-ordinated expression and activation of any of a number of latent MMPs. I have shown that MMP-2 does at least in part play a role in resolution of fibrosis. Active MMP-2 is not only present in resolving fibrotic liver, but it is able to degrade type I collagen that is present in the fibrotic bands (Aimes & Quigley 1995). Resolution of fibrosis is likely to be a highly organised series of molecular events (Friedman 2000) of which activation of latent MMP-2 is merely a part. In support of this I demonstrated that active MMP-9 also has a pro apoptotic effect in vitro albeit not as potent as active MMP-2. Kupffer cells are known to synthesise MMP-9 and may therefore contribute to matrix degradation (Winwood et al. 1995). Neutrophil derived elastase has been demonstrated to inactivate TIMP-1 from pro MMP-9/TIMP-1 complexes allowing enhanced activation of MMP-9 by MMP-3 (Itoh et al. 1995). Also, mast cell chymase has been shown to inactivate TIMP-1 and prevent it protecting pro MMP-9 from activation by other proteases (Frank et al. 2001). Together, these observations suggest that inflammatory cells have the potential to influence activity of MMPs and therefore

apoptosis of HSC. In the kidney, the macrophage has been shown to have a key role regulating loss of mesangial cells by apoptosis (Duffield et al. 2000). In the liver, the equivalent cells are the Kupffer cells and their role in regulating HSC apoptosis is currently under investigation. An interesting line of evidence in support for an important role in liver fibrosis of inflammatory cells comes from the study by Issa et al (2003). In this study, mice with collagen mutated (r/r collagen) to be resistant to degradation by MMPs (Krane et al. 1996) were used in an experimental model of liver fibrosis induced by carbon tetrachloride. Wild type mice underwent spontaneous resolution of fibrosis. In contrast, mice with mutant collagen did not show evidence of spontaneous resolution of fibrosis over the twenty eight day period of spontaneous recovery. An unanticipated finding was that in the mutant mice there was also a significantly increased inflammatory infiltrate in and around the bands of fibrosis (personal communication from Professor Iredale). This confirms that inflammation also plays a role in liver fibrosis and fibrosis resolution. Indeed, agents that that are anti inflammatory have been shown to be effective treatments for certain types of liver injury where inflammation plays a major role, for example corticosteroid therapy in chronic auto immune hepatitis (Dufour et al. 1997).

7.1.9 Together, the data described in this thesis provides strong evidence that TIMP-1 is mechanistically important in promoting fibrosis: Firstly by directly inhibiting MMPs thus promoting matrix accumulation, and secondly, by inhibiting the apoptosis of activated hepatic stellate cells via inhibition of MMP activity. Another important discovery from this work is that promotion of apoptosis of HSC is assocated with increased activation of MMP-2. This observation has importance in terms of therapeutic design of new agents to treat liver fibrosis. As I described in some detail in the introduction there are many approaches currently in development to treat liver fibrosis. Promotion of apoptosis is an attractive method to treat fibrosis for two reasons. Firstly, it would remove the cells responsible for synthesising collagen type I and TIMP-1 that inhibits collagen degradation. Secondly, I have shown apoptosis is a way of activating latent MMP-2. So, at least in principle, promoting apoptosis of HSC is a means of recruiting MMP-2 to participate in matrix degradation locally. These observations highlight the balance of active MMP-2 and TIMPs as an important potential therapeutic target in the treatment of liver fibrosis in the future.

7.2 The relevance of the work to our understanding of apoptosis

7.2.1 The role of MMPs in apoptosis- Apoptosis represents a highly organised cascade of proteolytic events involving caspases and many other enzymes (Hengartner 2000). The intracellular molecular biology of apoptosis is well characterised. In contrast, the extracellular molecular biology of apoptosis is less well descibed. Much is known about the families of receptors and their ligands that promote or facilate apoptosis. However, the understanding of the role of an individual MMP in apoptosis is sparse in the literature. Most publications that describe MMP dependent shedding of receptors or surface molecules have used data from MMP inhibitor studies that inhibit a broad range of MMPs (Herren et al. 1998). A natural model of apoptosis is the mammary gland epithelium. One study that made use of a transgenic mouse with an autoactivating MMP-3 (Stromelysin-1) demonstrated that mammary epithelial cells underwent unscheduled apoptosis during pregnancy. In contrast, mice that were bred to also have an excess of TIMP-1 did not show enhanced apoptosis (Alexander et al. 1996). This study provided proof of concept that the balance of TIMP-1 and MMP-3 affected mammary gland epithelial cell fate. The work I have described in my thesis shows similar basic observations that TIMP-1 prevents while MMPs promote apoptosis. The additional observation is that I have demonstrated that active MMP-2 on its own, albeit at a high concentration (10nM or 620ng/ml) promotes apoptosis while at lower concentrations it is a mitogen. Therefore, the biological response of exposure to active MMP-2 is at least in part concentration dependent. Another interesting observation was that the mitogenic effects of active MMP-2 were overcome if another mitogen was present (eg PDGF or IGF-1). It is therefore difficult to dissect out the dominant role MMP-2 has on HSC survival because the cellular response is dependent on context.

7.2.2 The role of TIMPs in cell surival - The current evidence suggests that TIMPs have divergent effects on proliferation and apoptosis in a number of different cell types apparently independent of their MMP inhibitory activity. TIMP-1 was originally described to have erythroid potentiating activity (Docherty et al. 1985). More recently, TIMP-1 has been shown to prevent apoptosis in breast epithelial cells (Li et al. 1999), B lymophocytes (Guedez et al. 1998) and mesangial cells (Lin et al. 2002). Overall, reports consistently find that TIMP-1 promotes cell survival. In contrast, studies of TIMP-2 describe conflicting effects. For example TIMP-2 appears to stimulate mesenchymal growth in the kidney (Barasch et al. 1999), inhibit apoptosis of melanoma cells (Valente et al. 1998) while it has been shown to

promote apoptosis of T lymphocytes (Lim et al. 1999). Studies of TIMP-3 have been more consistent suggesting that it has a predominantly proapoptotic effect in smooth muscle cells (Baker et al. 1998) and colonic carcinoma cells (Smith et al. 1997). The studies of TIMP-1 I have described in this thesis conclude that inhibition of HSC apoptosis by TIMP-1 is via MMP inhibition. There are two lines of evidence to support this conclusion. Firstly, studies with the synthetic MMP inhibitors suggested that MMP inhibition was likely to be the mechanism mediating survival of HSC. Secondly, the T2G mutant N-TIMP-1 studies demonstrate directly that inhibition of apoptosis of HSCs by TIMP-1 is in fact mediated via its effects on MMP activity. The T2G mutant N-TIMP-1 protein differs from the wild type protein by only a single amino acid substitution (Threronine to Glycine at amino acid position number 2) which reduces the inhibition constant of TIMP-1 for MMP-1, MMP-2 and MMP-3 by factors of 6000, 93,000 and 700 respectively (Meng et al. 1999). This makes it the best available reagent available to address the issue of MMP dependency in protection from apoptosis. At the dose of TIMP-1 used in my experiments (142.5ng/ml), the mutant TIMP-1 would have effectively no MMP inhibitory activity while the wild type TIMP-1 would be expected to significantly reduce MMP activity. The T2G mutant TIMP-1 is particularly poor at inhibiting MMP-2 further supporting a role for MMP-2 in apoptosis.

Further evidence to support the idea that MMP inhibition promotes cell survival comes from an elegant study by Ho et al. (2001). In this study, MMP inhibitors were demonstrated to increase fibroblast adhesion through stabilisation of focal adhesion contacts and up regulation of cadherin function. This study is complimented by the observations I made looking at N-cadherin and β-catenin during HSC apoptosis induced by gliotoxin and the effects of TIMP-1, TIMP-2 and the MMP-2 inhibitor. Together this work provides convincing data that the balance of TIMPs and MMPs influence cell survival by regulating the protection or degradation of cell survival stimuli.

7.3 Suggestions for future study

7.3.1 Determining the role of TIMP-1 in an experimental liver fibrosis recovery- An interesting in vivo model to determine the role of TIMP-1 on HSC survival would be to repeat a recovery model using TIMP-1 knockout mice. At the start of this work I had originally intended to use TIMP-1 knockout mice for this purpose. Recently, a transgenic mouse that overexpresses TIMP-1 has been used to demonstrate that TIMP-1 promotes liver fibrogenesis in animal model (Yoshiji et al. 2000) and so to some extent the field has moved on and

repeating a recovery model using the TIMP-1 knockout mice would be covering common ground albeit from a different angle. A more exciting line of study would be to move to more translational research perhaps attempting to target TIMP-1 using interference RNA techniques in a rat model of fibrosis.

7.3.2 Determining the role of MMP-2 in liver fibrosis – An obvious further line of study would be to determine the role of MMP-2 and TIMP-2 in an experimental model of liver fibrosis and spontaneous recovery from liver fibrosis. This could be achieved by establishing an experimental model of liver fibrosis using knockout mice. In particular a knockout of MMP-2. Such an experiment could clarify the role of MMP-2 in liver fibrogensis compared to spontaneous resolution of liver fibrosis. An alternative knockout model would be to use a TIMP-2 knockout mouse. TIMP-2 is critical for the activation of mature active MMP-2 and may therefore also behave like an MMP-2 knockout mouse. An interesting further line of study would be to examine the effect of certain agents (eg. Concanavalin A, plasmin and MT1 MMP/TIMP-2 complex) that are known to activate MMP-2. For example concanavolin A has been used to activate MMP-2 in vitro (Benyon et al. 1999). At low concentrations concanavalin A promoted proliferation of HSC but at higher concentration cells detached from the culture plates (personal communication from Dr R C Benyon). An interesting in vivo study would be to administer concanavolin A to a rat at peak fibrosis to assess whether activating MMP-2 in this manner would accelerate resolution of liver fibrosis.

7.3.3 Determining the mechanism of activation of MMP-2 that occurs with HSC apoptosis- Another interesting further study would be to examine the timing and events that lead to activation of MMP-2 during HSC apoptosis. My studies have not addressed a crucial question that follows this observation namely what happens first, activation of the caspase cascade or activation of MMP-2? It is entirely plausible that the mode of apoptosis is in fact anoikis, that is the cell must first detach itself from its surrounding microenvironment before the caspase activity occurs. This study could be relatively easily undertaken in vitro using a panel of caspase enzyme inhibitors and the Biotrak MMP-2 activity assay.

7.3.4 Determining the fate of TIMP-1 during apoptosis- An important question that has not been fully addressed is what happens to the TIMP-1 protein during apoptosis of HSC? I have presented some data suggesting that it is degraded during apoptosis. Greater understanding of the mechanisms of secretion and clearance of TIMP-1 from HSC might reveal novel methods of influencing TIMP-1 levels in vivo.

7.3.5 Determining the role of β-catenin signalling in HSC- The laser confocal studies clearly showed redistribution of β-catenin after induction of apoptosis by gliotoxin or active MMP-2. It would be interesting to further study the role of β-catenin in the regulation of HSC survival. In particular, it would be worth while to search for β-catenin responsive elements that might control MMP transcription. Recent evidence from studies of colon cancers suggests that the promotor for MMP-7 (or matrilysin) contain a responsive element to β-catenin/TCF-4 (Brabletz et al. 1999) providing proof of concept that β-catenin signalling may be involved in regulation of some types of MMPs.

Appendix I

List of solutions used:

Cell lysis buffer:	50mM HEPES
	1mM DTT
	0.1mM EDTA
	0.1% CHAPS
	pH 7.4
DEPC water	0.1% DEPC in water overnight then autoclave

Electrophoresis sample buffer:

	0.75ml deionised Formamide
	0.15ml 10x MOPS
	0.24ml Formaldehyde
	0.1ml DEPC water
	0.1ml Glycerol
	0.08ml 10% Bromophenol Blue.
Ethidium bromide	1mg/ml

Liver Homogenising buffer:	50nM	Tris HCl pH7.6
	0.25%	Triton X-100
	0.15M	NaCl
	10mM	$CaCl_2$
	0.1mM	Phenylmethylsulfonylfluoride
	10μM	Leupeptin
	10μM	Pepstatin A
	0.1mM	Iodoacetamide
	25μg/ml	Apoptonin

10x MOPS:	0.2M Morpholino propanosulfonic acid
	50mM sodium acetate
	10mM EDTA

0.1% DEPC adjusted to pH 7 using NaOH and autoclaved

5x Running buffer for western blotting:

9g Tris base

43.2g glycine

3g SDS all diluted in 600ml distilled water

2x Sample buffer for western blotting:

4.6ml water

1ml 0.5M Tris pH 6.8

0.8ul glycerol

1.6ml 10% SDS

0.6ml bromophenol blue.

Separating gel SDS PAGE (8%):

4ml Acrylamide

5ml 1.5M Tris pH 8.8

10ml distilled water

0.2ml 10% SDS

10µl TEMED and 100µl of 10% ammonium persulphate used to polymerise gel

Stacking gel SDS PAGE:

1ml Acrylamide

2.52ml 0.5M Tris pH 6.8

6.4ml distilled water

0.1ml 10% SDS

10µl TEMED and 50µl of 10% ammonium persulphate used to polymerise gel

4x Transfer buffer for western blotting:

12.12g Tris base

	57.6g glycine, these were diluted in 1 litre of distilled water. The final buffer was made by diluting the stock buffer 1 in 4 with 550ml water and 200ml methanol
1.5M Tris HCl pH 8.8:	18.17g Tris base 100ml distilled water, pH adjusted to 8.8 with HCl
0.5M Tris HCl pH 6.8:	6.06g Tris base 100ml distilled water, pH adjusted to 6.8 with HCl
20x Tris Buffer Saline	48.4g Tris base 234g Sodium Chloride in 1 litre of distilled water pH 7.4
TE	10mM Tris-HCl 1mM EDTA pH 8.0
0.1%TTBS	50ml 20x TBS 1ml Tween 20 950ml distilled water
Trypan blue	0.05% trypan blue

Appendix II

AII.1 Introduction - The 12 and 6 week carbon tetrachloride animal models of liver fibrosis

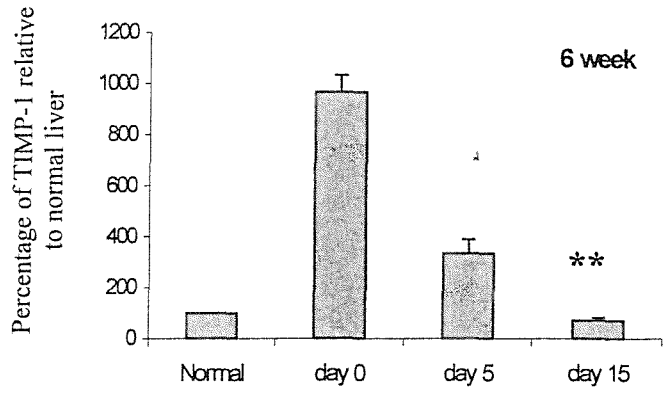
The background work to the project focus of the role of TIMP-1 in HSC survival was part of a wider collaborative venture in the laboratory to which I made a significant contribution between June 200 and May 2003. Work on in vivo models was undertaken between myself, Dr Xiaoying Zhou (Graduate student) and Dr Razao Issa (Postdoctoral fellow) supervised by Professor John Iredale and Dr Christopher Benyon.

AII.2 Persistence of TIMP-1 expression is accompanied by persistence of activated HSC and decreased resolution of liver fibrosis.

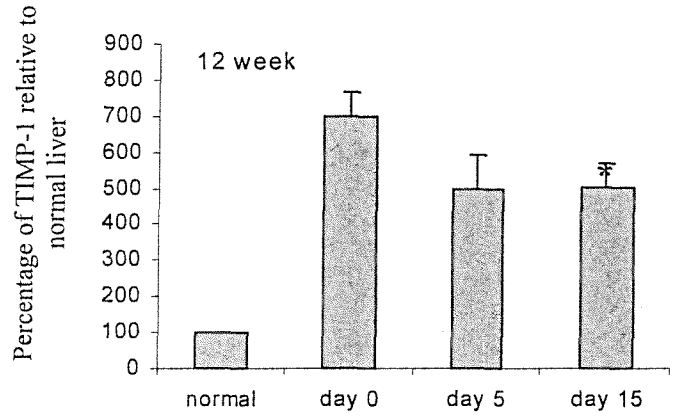
It has previously been demonstrated that TIMP-1 falls during spontaneous recovery of experimental fibrosis following 4 weeks of carbon tetrachloride intoxication (Iredale et al. 1998). To determine whether TIMP-1 mRNA remained elevated in liver cirrhosis, a further model of experimental fibrosis was undertaken. Rats injured with carbon tetrachloride as described in the methods were harvested after 12 and 6 weeks of intoxication and after a further 5 and 15 days of spontaneous recovery for each model. Taqman quantification of TIMP-1 mRNA was undertaken in the same livers with the help and supervision of Dr Xiaoying Zhou who designed the Taqman primers and probes for TIMP-1 and GAPDH. Over the 15 days of spontaneous recovery there was only a 2 fold decrease in expression of TIMP-1 mRNA after 12 weeks carbon tetrachloride (Figure AII.1a). Whereas during recovery there was a 13 fold decrease in TIMP-1 mRNA expression after 6 weeks of carbon tetrachloride (Figure AII.1b).

FIGURE AII.1A & AII.1B TAQMAN QUANTIFICATION OF TIMP-1 MRNA RELATIVE TO GAPDH IN 6 AND 12 WEEK MODELS OF RECOVERY FROM LIVER FIBROSIS

A



B



Figures AII.1a & AII.1b. TIMP-1 mRNA expression was determined by Taqman quantitative PCR in total liver RNA. After 6 weeks treatment with carbon tetrachloride a 13 fold decrease in TIMP-1 expression occurs during the first two weeks of spontaneous recovery (Figure AII.1a, PF0 cf. PF15, 6 week CCl_4). In contrast, there is only a 2 fold fall in TIMP-1 mRNA during the first 15 days of recovery in the 12 week injured rat liver (Figure AII.1b, PF0 cf. PF15, 12 week CCl_4). Data are presented relative to TIMP-1 expression in normal liver which has been given the arbitrary value of 100. All values have been normalised for GAPDH expression determined in parallel. (*$p<0.05$, **$p<0.005$ by Student's t test, PF0; peak fibrosis, immediately after the final injection of carbon tetrachloride, PF5 & PF15; after 5 & 15 days of spontaneous recovery. n=3 for each experiment group at each time point). Work was shared with Dr Xiaoying Zhou who has expertise with the Taqman technique.

AII.3 Immunohistochemistry for α-smooth muscle actin and cell counting.

To determine whether the persistence of TIMP-1 expression after 12 weeks carbon tetrachloride correlated with persistence of activated HSC and a failure of matrix degradation, immunohistochemistry for alpha smooth muscle actin and histological analysis were undertaken on the same livers. Staining of these liver sections for alpha smooth muscle actin was undertaken by Dr Razao Issa. Immunostaining of sections for alpha smooth muscle actin with cell counting and Western analysis of liver homogenates for alpha smooth muscle actin demonstrated that there was only a slight decrease in alpha smooth muscle actin positive activated HSC during recovery (Figure AII.2, AII.3). Indeed significant numbers of alpha smooth muscle actin positive activated HSC were present in the 15 day recovery livers after 12 weeks of carbon tetrachloride.

FIGURE AII.2 IMMUNOHISTOCHEMISTRY FOR α-SMOOTH MUSCLE ACTIN AND CELL COUNTING OF 6 AND 12 WEEK MODELS OF LIVER FIBROSIS.

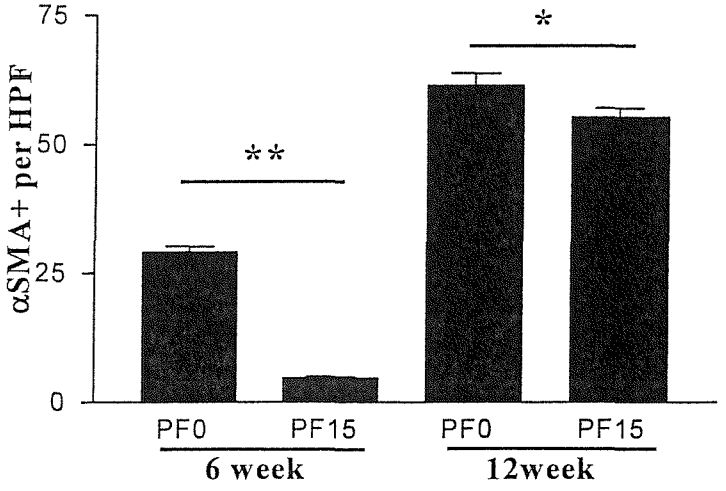


Figure AII.2. The numbers of alpha smooth muscle actin (αSMA) positive HSC were quantified in section form after 6 and 12 weeks of carbon tetrachloride intoxication and after 15 days of spontaneous recovery as described in the methods. During the first two weeks of spontaneous recovery from rat liver fibrosis there is minimal change in the number of alpha smooth muscle actin positive HSC in the 12 week injured liver (PF0 cf. PF15, 12 weeks) while in the liver injured for 6 weeks there is a dramatic decrease in the number of alpha smooth muscle actin positive staining cells (PF0 cf. PF15, 6 week). (Data presented are mean+/-SEM. PF0 = Peak fibrosis, immediately after the final injection of carbon tetrachloride, PF15 = after 15 days of spontaneous recovery, n=4 for each experimental group at each time point; **p<0.005, *p<0.05).

FIGURE AII.3 WESTERN BLOTTING FOR ALPHA SMOOTH MUSCLE ACTIN OF 6 AND 12 WEEK MODELS OF LIVER FIBROSIS

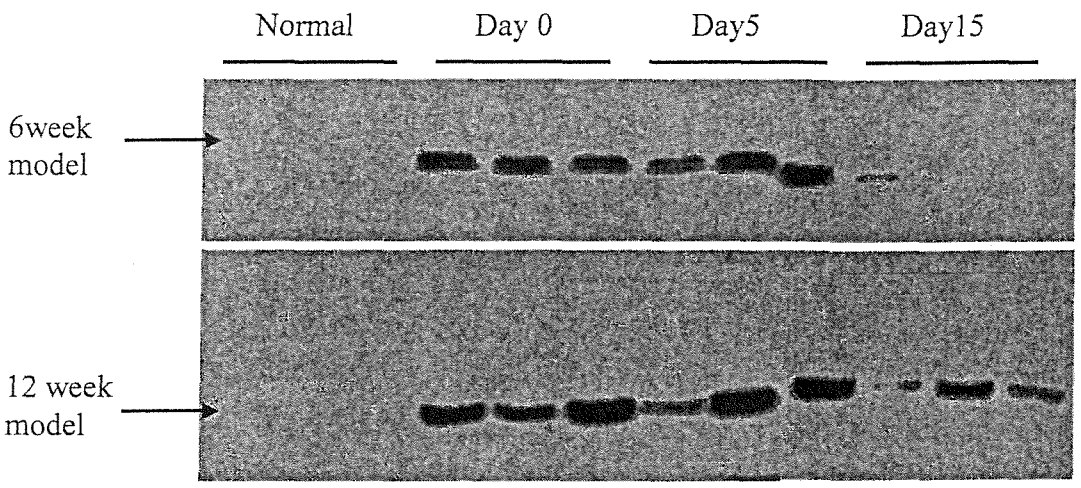


Figure AII.3. Western blotting of whole liver homogenate for alpha smooth muscle actin demonstrates reduction in levels of liver alpha smooth muscle actin protein over the first 15 days of recovery after 6 weeks of carbon tetrachloride intoxication (Day 0 cf. Day 15, 6 weeks CCl_4). In contrast, levels of alpha smooth muscle actin protein remain elevated in whole liver extracts from the animals injured with carbon tetrachloride for 12 weeks even after 15 days of spontaneous recovery (Day 0 cf. Day 15, 12 weeks CCl_4). In both models the liver alpha smooth muscle actin protein level is increased at peak fibrosis (Day 0) relative to normal livers (Normal). (Normal: untreated liver control, Day 0: immediately after the final injection of carbon tetrachloride, Day 5 and Day 15 after 5 and 15 days of spontaneous recovery respectively; n=3 for each time point). Western blot was undertaken by Dr Xiaoying Zhou.

AII.4 The 12 and 6 week carbon tetrachloride model histology collagen by assessed by sirius red staining

By Sirius red staining, the 12 week carbon tetrachloride model had histological evidence of an established cirrhosis at peak fibrosis (Figure AII.4a) and demonstrated only modest remodelling over the 15 day spontaneous recovery period (Figure AII.4b). In contrast, after 6 weeks of carbon tetrachloride, the numbers of alpha smooth muscle actin positive HSC demonstrated a highly significant decrease (Figure AII.4c, AII.4d). Indeed alpha smooth muscle actin expression was not detectable by western analysis in the 6 week treated livers after 15 days recovery (Figure AII.3). Histological analysis of the 6 week model peak fibrosis samples demonstrated an established septal fibrosis (Figure AII.4c). Over the 15 days of spontaneous recovery there was evidence of significant matrix remodelling (Figure AII.4d). The remodelling of fibrosis in the 6 week model during 15 days recovery was accompanied

with a 50 percent drop in liver hydroxyproline content to a level identical to that seen in untreated control liver. In contrast, the 12 week model showed increased levels of hydoxyproline of 150 percent of normal liver at peak fibrosis, which did not significantly change over the 15 days of spontaneous recovery.

FIGURE AII.4. THE 12 AND 6 WEEK CARBON TETRACHLORIDE MODEL HISTOLOGY ASSESSED BY SIRIUS RED STAINING

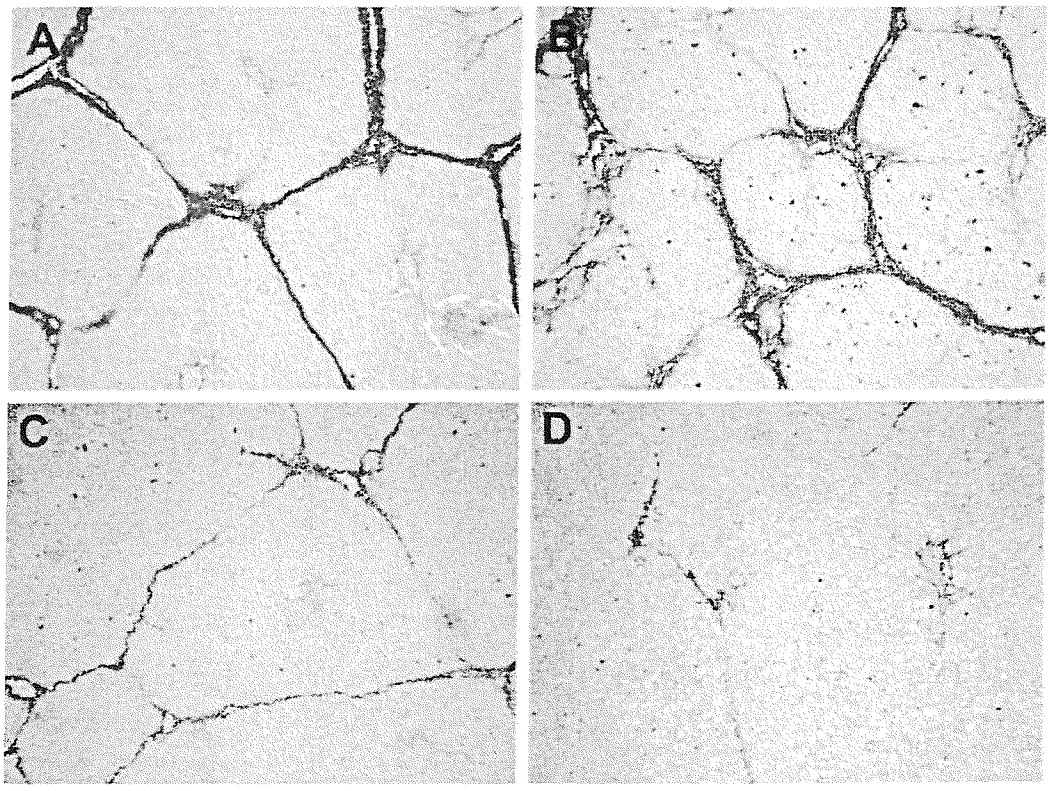


Figure 3.4a-d. Histological analysis (Sirius red stain by the histopathology department of Southampton University) of rat livers harvested after 6 and 12 weeks of carbon tetrachloride intoxication twice weekly as described in the methods. Livers were harvested at peak fibrosis (PF0) following 12 (plate A) and 6 (plate C) weeks treatment and after a further 15 days of spontaneous recovery (plates B and D respectively). In the 12 week model there is more substantial fibrosis (indeed cirrhosis is present) compared to the 6 weeks of injury (plates A and C respectively). Furthermore, there is evidence of only modest matrix remodelling during the 15 days of spontaneous recovery in the 12 week model (plates A to B). In the 6 week model there is an established septal fibrosis present (plate C), which demonstrates evidence of remodelling over 15 days (plate C to D).

AII.5 Collagenase activity increases during recovery from liver fibrosis

To determine whether the observed changes in TIMP-1 mRNA expression were associated with MMP inhibition, collagenase activity in whole liver homogenate was undertaken. This demonstrated that after 12 weeks of carbon tetrachloride, at no time point (days 0,5 or 15 days

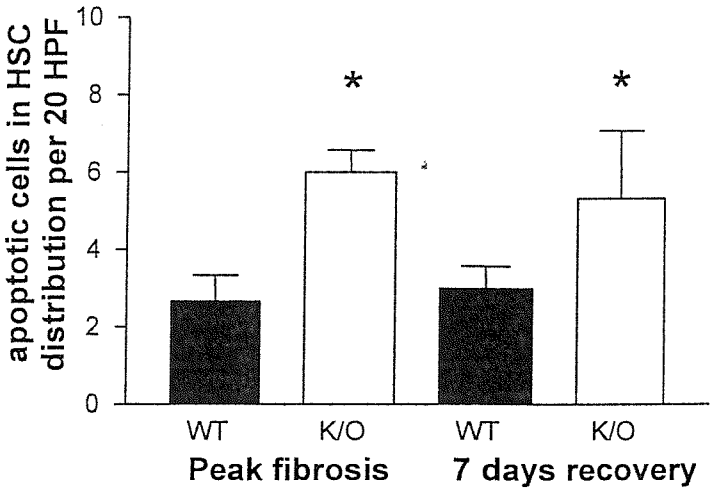
of recovery) was activity above that seen in normal untreated liver (collagenase activities expressed as percentage normal liver (+/-SEM) were: day 0: 70% (+/-1.9%), day 5: 60% (+/-3.3%), day 15: 55% (+/- 3.7%)). In contrast, after 6 weeks of carbon tetrachloride, collagenase activity in the liver homogenates demonstrated an increase peaking at 5 days of recovery (collagenase activities expressed as percentage normal liver (+/-SEM) at each time point were: day 0: 70% (+/-1.9%), day 5: 147% (+/-3.3%), day 15: 107% (+/-1.6%). Collagenase assays were performed by Dr Xaioying Zhou (Postdoctoral fellow). Together these data demonstrate a strong correlation between persistence of activated HSC following fibrotic injury and TIMP-1 expression and a failure of matrix degradation with persistent inhibition of collagenase activity.

AII.6 TIMP-1 knockout model of liver fibrosis recovery

Enhanced DNA fragmentation is observed in the TIMP-1 knockout mouse compared to the wild type- To determine the in vivo significance of TIMP-1 and liver fibrosis, a mouse model of carbon tetrachloride induced fibrosis was undertaken previously by the liver group of Southampton University in 1998 comparing wild type animals to TIMP-1 (-/-) knockout mice. Interestingly, there was no significant difference in levels of fibrosis comparing TIMP-1 knockout and the wild type (unpublished data). Archival material from this model was used in a pilot study to determine the importance TIMP-1 on HSC survival. Livers from cohorts of mice at time points (0 to 7 days) after the final injection of carbon tetrachloride were used. TUNEL staining and counting of apoptotic HSC and hepatocytes was undertaken by a blinded observer. While comparison of TIMP-1 knockout to wild type animals at the individual time points only showed a trend (that was not statistically significant), combining the time points and using a one way analysis of variance did show a significant difference. That is that the TIMP-1 knockout mouse HSC showed more apoptosis compared to the wild type (Figure AII.5a). This contrasted with the parenchymal cell which showed no difference in levels of DNA fragmentation (Figure AII.5b).

FIGURE AII.5A & AII.5B. COMPARISON OF DNA FRAGMENTATION OBSERVED IN TIMP-1 (-/-) KNOCKOUT MICE COMPARED TO WILD TYPE CONTROLS FOR HSC AND PARENCHYMAL CELLS.

A



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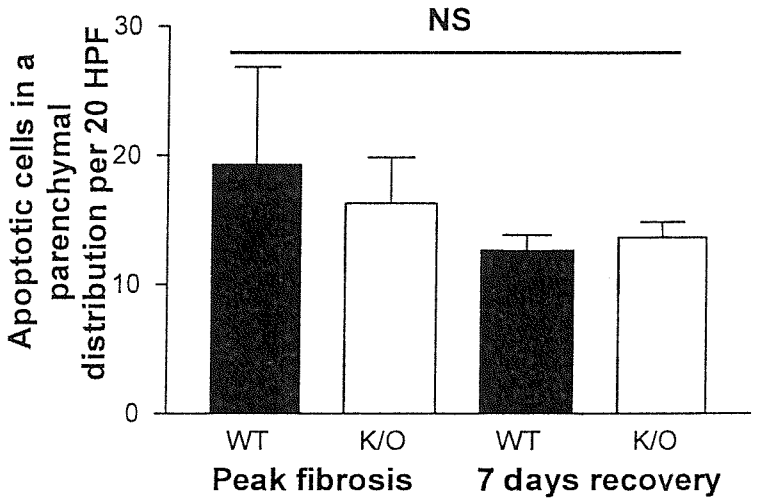

Figure AII.5a & AII.5b. Mice injured with carbon tetrachloride which were allowed to spontaneously recover over 7 days showed different levels stellate cell DNA fragmentation assessed by the TUNEL technique (performed by Dr Razao Issa) when the TIMP-1 knockout mice were compared to their wild type controls (Figure AII.5a, *$p<0.05$). In contrast, there was no difference in parenchymal cell DNA fragmentation assessed by the TUNEL technique when TIMP-1 knockout was compared to their wild type controls (Figure AII.5b).

AII.7 Summary of the in vivo findings

1. Persistence of TIMP-1 expression is accompanied by persistence of activated HSC and decreased resolution of liver fibrosis.
2. Decrease in TIMP-1, increase in collagenase activity and resolution of fibrosis are associated with increased HSC apoptosis
3. There is increased apoptosis in HSC in the TIMP-1 knockout mice model of recovery implicating TIMP-1 as an anti apoptotic factor for activated HSC but not hepatocytes.
4. Together, this data support a role for TIMP-1 as an anti apoptotic factor for activated HSC in vivo.

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