Hepatic lipocytes, TIMP-1 and liver fibrosis

ABSTRACT—In progressive liver fibrosis, the role of extracellular collagen deposition exceeds its rate of degradation. Collagen and related proteins are synthesised in the fat-storing liver cells (lipocytes). When injured, these cells proliferate and change into myofibroblast-like cells, secreting even more collagen into the extracellular space. The degradation of collagen is accomplished by metalloproteinases, whose activity is reduced by tissue inhibitors (TIMPs). Injured lipocytes produce an excess of these inhibitors. The final result of lipocyte injury is thus progressive liver fibrosis. There is evidence that TIMPs also play a role in progressive fibrosis in other tissues.

Cirrhosis is a major worldwide health care problem. In the United Kingdom alone it currently causes more than five thousand deaths each year. Cirrhosis may be caused by chronic liver disease of any aetiology and is the end result of a protracted time course of progressive liver fibrosis. The global importance of chronic liver disease and cirrhosis, coupled with recent advances in cell and molecular biology, has catalysed renewed investigation of the cellular and molecular events that characterise liver fibrosis.

Hepatic lipocytes, also called fat-storing or Ito cells, are pivotal to the pathogenesis of liver fibrosis (Fig 1). In normal liver, these cells are situated in the subendothelial space of Disse and are the major storage site of retinoids (as retinyl esters) [1,2]. In areas of liver injury, lipocytes proliferate, lose their retinoid storage droplets and adopt a myofibroblast-like phenotype [3,4]. These cells are the major source of collagens and other matrix proteins deposited in fibrotic liver [4]. In animal models and in human liver disease, *in situ* hybridisation studies have demonstrated that mRNA transcripts for collagen types I and III are exclusively confined to these cells [5–7]. These observations are supported by biochemical studies of cultured lipocytes. If plated on either collagen I or plastic, lipocytes adopt a myofibroblast-like morphology and secrete collagens (predominantly types I and III) [8], laminin [9], proteoglycans [10,11] and fibronectin [12].

While synthesis and assimilation of matrix proteins is clearly important, another potential component of the pathogenesis of liver fibrosis is an alteration in the rate of extracellular matrix degradation. One emerging concept is that failure to degrade the excess fibrillar collagens laid down in fibrotic liver may be an important contributory factor to the progression of this process. This article presents the evidence for this suggestion, together with a brief review of the enzymes involved in extracellular matrix degradation, the matrix metalloproteinases, and their specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs).

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Matrix metalloproteinases

The matrix metalloproteinases are a family of closely related enzymes that exhibit degradative activity against a broad range of extracellular matrix proteins. They are important in a wide diversity of biological processes including organogenesis, morphogenesis, regeneration and wound healing/repair, and have a role in neoplasia/metastasis, connective tissue destruction and progressive fibrotic diseases.

So far nine members of this gene family have been identified; each of them encodes for an enzyme with a specific substrate profile for matrix (and other) proteins. The current nomenclature for these enzymes and their substrate profiles are summarised in Table 1. In brief, they are subdivided into three groups: *collagenases* which degrade interstitial collagens (types I, II and III); *gelatinases* which degrade basement membrane (type IV) collagen and gelatins (denatured collagens); and *stromelysins* which degrade a broad range of substrates including proteoglycans, laminin, gelatins and fibronectin.

The collagens that are deposited in fibrotic liver are the fibrillar interstitial collagens (predominantly types I and III) [4]. The most relevant enzyme (Table 1) for their degradation is *interstitial collagenase.* It was first described by Gross and Lapiere [13] in studies of tadpole tails undergoing metamorphosis. Human interstitial collagenase has now been cloned and sequenced [14] and its biochemical properties defined [15,16]. Its most important activity is its ability to cleave native collagens types I and III, thus initiating their degradation. Cleavage occurs at a specific Gly–Ile bond forming characteristic TC^A and TC^B fragments of 1/4 and 3/4 of the original collagen molecule, respectively. These cleavage products partially denature and become susceptible to further degradation by other proteinases, including the gelatinases (Table 1).

The extracellular activity of matrix metalloproteinases is tightly regulated, presumably because of their potent ability to degrade matrix. For all family mem-

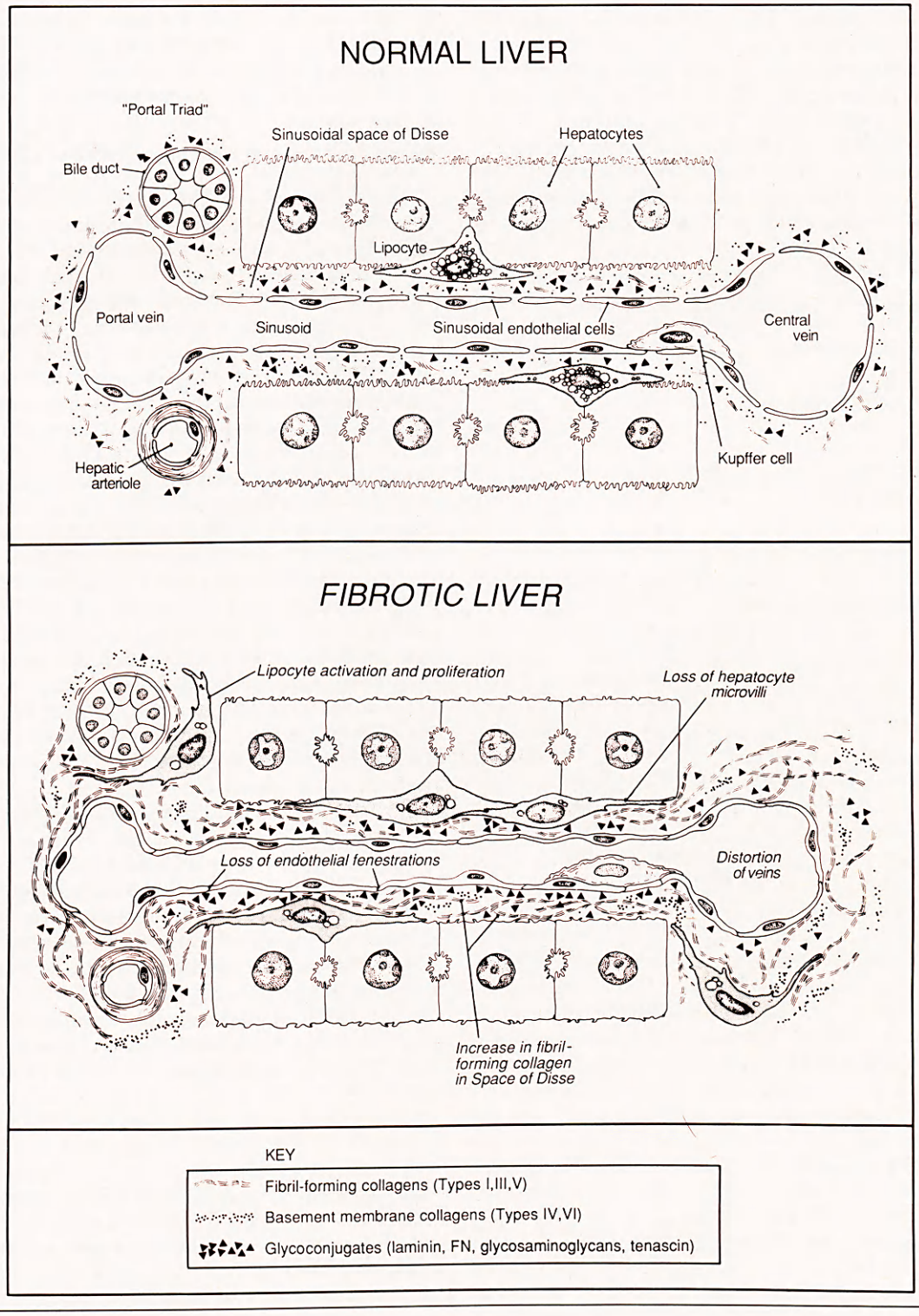


Fig 1. *Lipocyte activation and matrix alterations in liver fibrosis.* In normal liver, hepatic lipocytes are situated in the subendothelial space of Disse, which also contains basement membrane-like matrix proteins and a small amount of fibril-forming collagens. In fibrotic liver, lipocytes proliferate and transform to a myofibroblast-like phenotype with increased deposition of fibrillar collagens in the space of Disse. Figure kindly provided by Dr S L Friedman (Liver Center Lab, University of California, San Francisco) and reproduced from *Wright's liver and biliary disease* with the permission of Baillière Tindall (London).

bers, including interstitial collagenase, there are several different mechanisms for regulating the extracellular activity of these enzymes. They include changes in the rate of gene transcription and proenzyme synthesis, extracellular activation of secreted proenzymes to catalytic forms, prevention of proenzyme activation, and specific inhibition of activated forms by tissue inhibitor(s) of metalloproteinases (TIMPs). It is beyond the scope of this article to review each of these mechanisms in detail but, in view of data obtained from studies of cultured hepatic lipocytes and human liver fibrosis (see below), particular emphasis will be given to TIMPs and their dual role in both extracellular inhibition of active metalloproteinases and prevention of prometalloproteinase activation.

Table 1. Nomenclature and substrate specificity of metalloproteinases

Nomenclature	Substrate profile
Collagenases	
Interstitial collagenase	III > I, II, VII, X
Neutrophil collagenase	I > III, II
Gelatinases	
Gelatinase A (72 kDa type IV collagenase)	IV, ?V, VII, X, gelatin
Gelatinase B (92 kDa type IV collagenase)	IV, V, gelatin, ?III
Stromelysins	
Stromelysin-1 (Proteoglycanase, transin in rat)	III, IV, V, IX, laminin, proteoglycans, fibronectin, casein
Stromelysin-2	As for stromelysin-1
Matrilysin (PUMP-1)	IV, proteoglycans, fibronectin, gelatins, elastin
Stromelysin-3	Not known
Others	
Metalloelastase	Elastin, fibronectin

Roman numerals refer to collagen subtypes.

Tissue inhibitors of metalloproteinases (TIMPs)

Three members of the TIMP family have been described: TIMP-1, TIMP-2 and TIMP-3 or ChIMP. TIMP-1 and TIMP-2 have been characterised in detail (for recent detailed review see Murphy and Docherty [17]) but TIMP-3 has been more recently described [18,19]. Each TIMP is the product of a separate gene but there is 40% sequence homology between TIMP-1 and TIMP-2. These inhibitors exhibit their own distinct properties and differ in their regularity pathways but have also many similarities in structure and function. A comparison of the biochemical properties of the TIMPs is given in Table 2. In brief, TIMP-1 is a 30 kDa glycoprotein whereas TIMP-2 is a 23 kDa non-glycosylated protein. Both have 12 highly conserved cysteine residues forming disulphide bridges that confer a stable three loop structure [17,20]. Both TIMP-1 and TIMP-2 inhibit active metalloproteinases in a stoichiometric manner and there are no major differences in their ability to inhibit members of this enzyme family. TIMPs exert their effect by binding to the catalytic site of these enzymes, effectively inhibiting their degradative activity [21,22], but the precise mechanism for this effect is poorly understood [17]. It has been further analysed by engineering truncated forms of TIMPs and by site-directed mutagenesis of TIMP-1 in particular. These studies have demonstrated that the N-terminal portion of the molecule, particularly the anchored sequence between cysteine 3 and 13 (amino acids 1–126), is responsible for inhibitory activity against metalloproteinases, whereas the C-terminal portion is more important for binding to the C-terminal domain of prometalloproteinases [23–25]. TIMP-1 and TIMP-2 can also inhibit or prevent the extracellular activation of prometalloproteinases. This was first described for TIMP-2 which binds to the C-terminal domain of pro-

Table 2. Properties of TIMPs

	TIMP-1	TIMP-2	TIMP-3
Molecular size	30 kDa	23 kDa	21 kDa
Glycosylation	Yes	No	No
Metalloproteinase inhibition	All MMPs	All MMPs	Not known
Special features	Binds to progelatinase B	Binds to progelatinase A	Found exclusively in the ECM
Effect of TGF-β1	↑ gene expression	↓ gene expression	Not known

MMP, matrix metalloproteinase; ECM, extracellular matrix.

TIMP-3 or ChIMP-3 has only been described in chicken (to date).

gelatinase A and is often secreted from cells as a proenzyme–inhibitor complex [26–28]. When bound at this site, TIMP-2 inhibits progelatinase A activation by preventing its autocleavage in association with a cell membrane related mechanism [26,27,29]. Other mechanisms of regulating proenzyme activation also occur for other metalloproteinases; for example, TIMP-2 binds to interstitial procollagenase, thus inhibiting its proteolytic activation [30], and TIMP-1 binds to progelatinase B, forming a complex that cannot be activated by either plasmin or stromelysin [31]. Thus, by a combination of binding to either the C-terminal domain or the catalytic site, TIMPs exert a dual effect on regulating extracellular metalloproteinase activity.

Expression of the genes for TIMP-1 and TIMP-2 is regulated by many of the same growth factors and cytokines that regulate metalloproteinase gene expression. There may be co-regulation, eg EGF and b-FGF increase both interstitial collagenase and TIMP-1 gene expression, or inverse co-regulation, eg TGF-β1 decreases interstitial collagenase and TIMP-2 gene expression but increases expression of TIMP-1 [32–35]. The latter is of particular relevance to liver fibrosis as activated hepatic lipocytes autoexpress TGF-β1 [36] and TGF-β1 receptors [37]. The effect of retinoids is also likely to be pertinent as these are released from storage droplets during lipocyte activation [38]. They exert profound effects in other mesenchymal cells by increasing the expression of TIMP-1 and decreasing that of interstitial collagenase [39].

Collagenase activity and liver fibrosis

In progressive liver fibrosis, the rate at which fibrillar collagens are synthesised and laid down in the extracellular space exceeds the rate of degradation by the relevant metalloproteinase (interstitial collagenase), thereby leading to net accumulation of collagen. Alterations in either the rate of synthesis or the rate of degradation, or both, could contribute to this process.

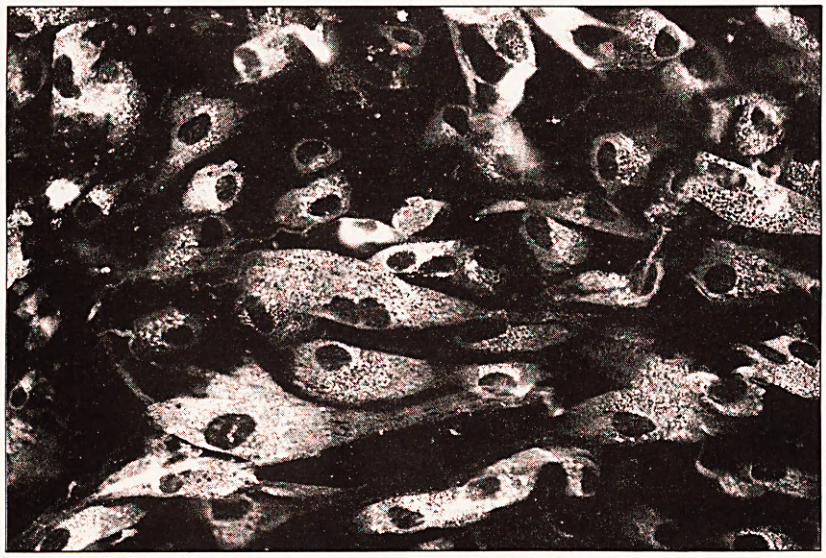
For studies of matrix degradation, the original observation was that liver explants obtained from CCl_4-treated rats degrade a type I collagen substratum more readily than explants from normal liver [40]. As this model is difficult to interpret, there have been many subsequent attempts to study interstitial collagenase activity in progressive liver fibrosis *in vivo*. Results have been widely divergent but a broad overview indicates that interstitial collagenase activity decreases as liver fibrosis progresses. This has been demonstrated in CCl_4-induced liver fibrosis in rat [41] and in advanced alcoholic cirrhosis in baboons and man [42,43].

There are several possible explanations for these observations including: decreased procollagenase biosynthesis, decreased procollagenase activation, or specific inhibition of activated collagenase. Some information on the interactions that can occur between these factors in liver fibrosis is provided by studies of experimental murine schistosomiasis [44–46]. In this model, hepatic interstitial collagenase activity was maximal after eight weeks, with increased release of enzyme. As infection progressed beyond eight weeks, interstitial collagenase activity fell, owing in part to decreased interstitial collagenase synthesis but also to its increased binding to alpha-2 macroglobulin which forms inactive enzyme–inhibitor complexes [47].

The concept that fibrosis progresses as a consequence of relative failure of matrix degradation has been the focus of our research. We have studied the relative expression and synthesis of interstitial collagenase and its major inhibitor, TIMP-1, in cultured hepatic lipocytes, in human liver disease and in animal models of liver fibrosis. This work will be discussed in relationship to other contributions to this and related fields of research.

Studies of TIMP-1 and interstitial collagenase in cultured liver cells

TIMP-1. Because of the overall importance of hepatic lipocytes to the pathogenesis of liver fibrosis and our earlier observation that these cells degraded matrix by release of gelatinase A [48], we have studied their ability to synthesise the metalloproteinase inhibitors TIMP-1 [49] and TIMP-2. These studies have immunolocalised TIMP-1 protein to primary cultures of human hepatic lipocytes (Fig 2a) and have also demonstrated, by northern blotting, that these cells express mRNA for TIMP-1. Cellular secretion of TIMP-1 into human lipocyte culture medium was demonstrated by ELISA, and the ability of secreted TIMP-1 to inhibit metalloproteinase activity was confirmed by reverse gelatin zymography (Fig 3). This technique revealed no evidence of TIMP-2 secretion by cultured human lipocytes. The quantitative importance of TIMP-1 secretion by hepatic lipocytes was demonstrated by separating this inhibitor from metalloproteinases in culture medium using gelatin–sepharose chromatography. This resulted in a 20-fold increase in detectable metalloproteinase activity, which could be re-inhibited by addition of column fractions containing the eluted TIMP-1. Of major importance was the observation that freshly isolated human hepatic lipocytes did not contain mRNA for TIMP-1, but that expression increased with duration of cell culture, as these cells transformed to a myofibroblast-like phenotype [49]. As this cellular phenotype is associated with hydrolysis of stored retinyl esters and release of retinoids [38], we have also investigated the effect of exogenous retinoids on TIMP-1 mRNA expression by cultured lipocytes and found this to be upregulated by retinoic acid (10^{-6}M) [50]. Taken together, these findings indicate that cultured human hepatic lipocytes express TIMP-1, secrete this inhibitor at a functionally significant concentration, and modulate TIMP-1 expression in response to retinoids and other factors



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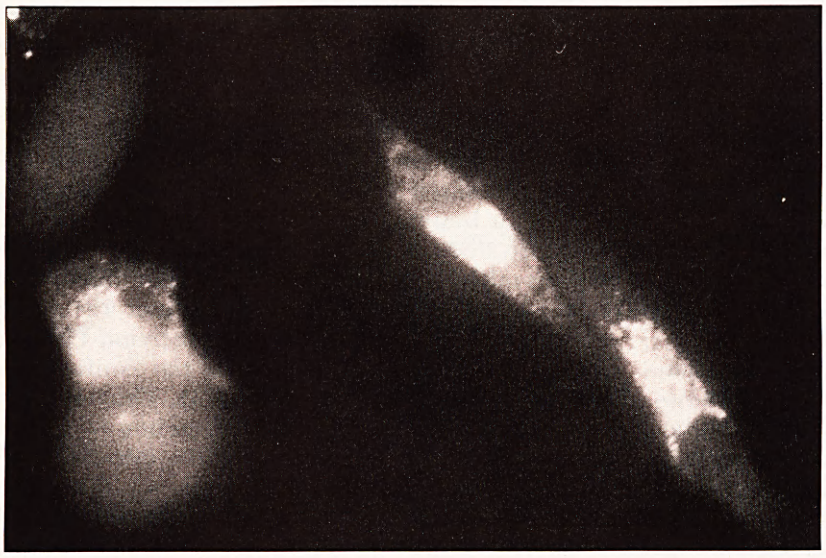

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Fig 2. *Immunolocalisation of TIMP-1 and interstitial collagenase in cultured human hepatic lipocytes.* Human lipocytes in primary culture were incubated in 5 μmol/l monensin for 3 h at 37°C and immunostained using either (a) anti-human TIMP-1 or (b) anti-human interstitial collagenase antibodies (kindly provided by Dr R Hembry and Dr G Murphy, Strangeways Research Laboratory, Cambridge). Figure 2b is reproduced with the permission of Kluwer Academic Publishers.

(yet to be defined) that promote cellular activation to a myofibroblast-like phenotype.

There is currently relatively little information on other cellular sources of TIMP-1 in liver. Recent work has demonstrated that HepG2 cells (a human hepatoblastoma cell line) express mRNA for, and synthesise, TIMP-1 and that this could be upregulated by IL-6, TGF-β1 and phorbol ester [51]. As many tumours express metalloproteinases and TIMPs, the relevance of this observation to normal or fibrotic liver is unclear. However, the same authors demonstrated that TIMP-1 mRNA was increased in whole rat liver after intraperitoneal injection of lipopolysaccharide and by cultured rat hepatocytes incubated with IL-6. This suggests that TIMP-1 may also be expressed by hepatocytes as part of an acute phase response. The data are potentially flawed because exhaustive steps are needed to purify rat hepatocyte cultures which otherwise contain significant numbers of hepatic lipocytes [52]. In the absence of immunolocalisation or *in situ* hybridisation data, the suggestion that hepatocytes can synthesise TIMP-1 remains unproven. Our experience of immunolocalisation of TIMP-1 in human liver biopsy specimens (detailed below) failed to reveal hepatocellular staining in normal, inflammatory or fibrotic liver.

Interstitial collagenase. Previous studies suggested that Kupffer cells were the cellular source of interstitial col-

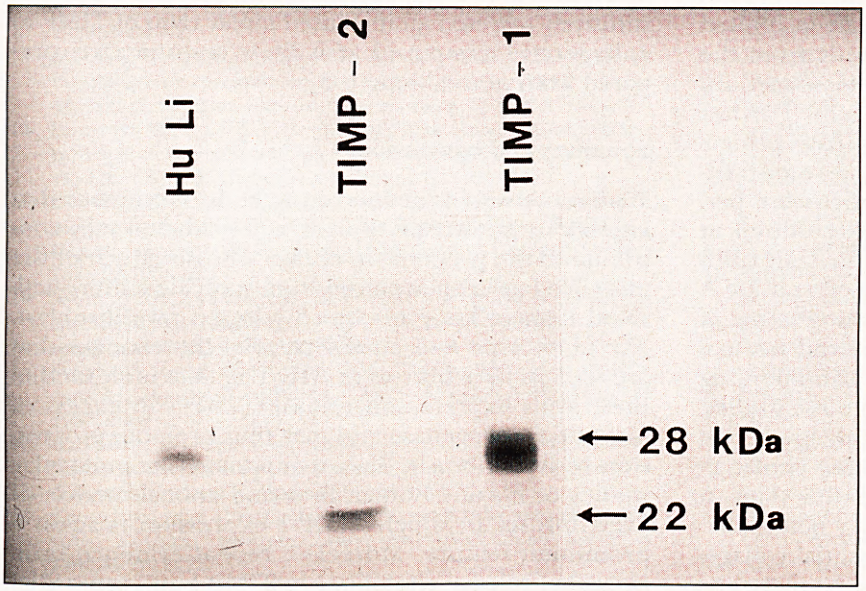

Fig 3. *Release of TIMP-1 activity by human lipocytes.* Reverse gelatin zymogram of human lipocyte medium (HuLi), with positive controls for TIMP-1 and TIMP-2. Human lipocyte medium contains a single band of metalloproteinase inhibitory activity that co-migrates with TIMP-1.

lagenase in liver [53,54]. With the development of specific antibodies, molecular probes, and improved techniques for lipocyte and Kupffer cell culture, hepatic lipocytes have now been shown to be the source of interstitial collagenase. Evidence for this was first obtained from studies of fibroblast-like cells (of possible lipocyte origin) prepared by outgrowth from a single human liver sample and exposed to IL-1 or TNF-α. These cells expressed mRNA for interstitial collagenase and released interstitial collagenase activity [55]. In subsequent studies we have immunolocalised interstitial collagenase to the cytoplasm of human lipocytes in primary culture (Fig 2b), whilst others have detected mRNA for this enzyme by northern analysis [56]. Our data demonstrate that relatively early primary human lipocyte cultures (four days) express mRNA for interstitial collagenase but this is suppressed in late primary culture or passaged lipocytes unless they are exposed to cytokines such as TNF-α (unpublished observations). TGF-β1 has recently been shown to downregulate interstitial collagenase expression by cultured human lipocytes [18]. In preliminary studies we have compared both mRNA expression and release of interstitial collagenase and TIMP-1 activity by lipocytes cultured with and without TNF-α. The striking observation is that, although interstitial collagenase mRNA expression increases, so does mRNA for TIMP-1 [57]. The net effect on released activity is neutral, with an excess of TIMP-1 and no detectable interstitial collagenase activity. Cultured *rat* hepatic lipocytes have also been reported to release interstitial collagenase activity when exposed to polyunsaturated lecithin [58], which may explain the antifibrogenic effect of this compound.

Studies of TIMP-1 and interstitial collagenase expression in fibrotic liver

The results obtained with culture-activated human hepatic lipocytes led to the hypothesis that TIMP-1 expression may, by inhibiting interstitial collagenase, play a significant role in the progression of liver fibrosis. Because of the technical difficulties posed by trying to investigate this hypothesis in an intact solid organ, several different approaches have been adopted.

Immunohistochemistry of human liver biopsy samples was hampered by the fact that there is no intracellular storage of metalloproteinases or TIMPs. Fresh liver biopsies were therefore incubated in monensin to acidify the cellular Golgi and so prevent export of synthesised proteins and create artificial storage conditions. Using this technique there was poor preservation of liver architecture, but some information was derived [59]. First, TIMP-1 protein could not be detected in normal liver, while interstitial collagenase was detected in only one of five normal livers. Second, in diseased human liver, all staining for metalloproteinases and TIMPs was associated with sinusoidal liver cells and not hepatocytes. Third, staining for TIMP-1 was found in six of 15 diseased livers whereas staining for interstitial collagenase was found in only four of 15, in three of which TIMP-1 was co-expressed.

While this information relates to the relevant proteins, its subjective nature and lack of quantitation lead to difficulties with interpretation. Further analysis of mRNA expression for either TIMP-1 or interstitial collagenase by northern analysis in total (or poly-A enriched) RNA from human liver failed to reveal positive signals despite positive control data. This indicates

that northern analysis lacked the necessary sensitivity to detect low levels of specific mRNA of interest (from lipocytes), presumably because this had been diluted in total liver RNA obtained predominantly from the more numerous hepatocytes (which also contain approximately 20-fold more RNA per cell). Further analysis was therefore performed by RNAase protection assay, which is estimated to be 100 times more sensitive than northern blotting. These studies were performed on snap-frozen liver explants obtained at transplantation from the Birmingham Liver Unit (with the kind cooperation of Dr J Neuberger and Dr A Strain). These samples were thus a combination of end-stage livers with advanced cirrhosis and normal donor human liver (not used for transplantation, eg reduced size graft for child recipient). In these studies, TIMP-1 mRNA expression was significantly greater in primary biliary cirrhosis, primary sclerosing cholangitis and autoimmune chronic active hepatitis than in normal human liver in which it was barely detectable. In contrast, low levels of mRNA for interstitial collagenase were found in normal human liver and there was no significant change in expression in either primary biliary cirrhosis or sclerosing cholangitis. There was a significant increase in interstitial collagenase expression in autoimmune chronic active hepatitis, but this was paralleled by an increase in TIMP-1 mRNA expression.

We have now complemented these data in humans with preliminary studies of the chronology of TIMP-1 expression in the rat model of CCl_4-induced liver injury and fibrosis. These experiments were performed using a rat TIMP-1 cDNA probe prepared by homology PCR (Dr W Ferris, details to be published elsewhere) and RNAase protection analysis. In this model, TIMP-1 mRNA expression increases within 24 hours of CCl_4 administration, peaks at 72 hours (5-fold that of control) and persists with continued dosing as the liver becomes fibrotic.

TIMP-1 and fibrosis in other tissues

The basic cellular and molecular pathogenesis of fibrosis in liver is unlikely to differ to any major degree from progressive fibrosis in other tissues. At present there is relatively little information about expression of TIMP-1 in other solid organs, but the available information is similar to that reported for liver. TIMP-1 mRNA expression was markedly increased in the New Zealand Black/White F1 murine model of lupus nephritis (in which there is accumulation of extracellular matrix in glomeruli) [60]. Similar data have recently been reported in human glomerulosclerosis; using competitive RT-PCR, TIMP-1 and TIMP-2 cDNA were detected in normal human glomeruli but were increased 4-fold in glomeruli obtained from glomerulosclerotic kidneys [61]. In studies of idiopathic pulmonary fibrosis, collagenase inhibitory activity was greater in whole lung homogenates prepared from fibrotic lung than in those from normal lung [62]. Moreover, fibroblasts prepared from fibrotic lung constitutively secreted an increased molar ratio of TIMP to collagenase compared with their counterparts prepared from normal lung [63].

Summary and conclusions

We have reviewed the increasing body of evidence that alterations in the rate of matrix degradation may contribute to the progression of liver fibrosis. Much of the evidence has been acquired from studying culture-activated hepatic lipocytes which adopt a myofibroblast-like phenotype and closely parallel the changes that are seen in liver fibrosis *in vivo*. The data indicate that these cells express and release TIMP-1 in excess of interstitial collagenase and may thus promote progression of liver fibrosis. This is supported by molecular studies of fibrotic human liver and animal models of liver fibrosis, in which TIMP-1 expression is again a prominent feature. Moreover, recent evidence indicates that relative alterations in TIMP-1 and interstitial collagenase may also be relevant to the pathogenesis of fibrosis in kidney and lung. In liver, much of this work is currently descriptive, with the mechanistic aspects implied rather than conclusively proven. The latter may be accomplished by application of modern molecular techniques, for example gene knockout technology, which may allow us to determine whether TIMP-1 expression is an *important* component of the pathogenesis of progressive liver fibrosis.

Acknowledgements

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The 1994 BUPA prizes for medical writing and illustration

Dr Hugh Thelwall-Jones, BUPA Group Medical Director, presented the 1994 BUPA prizes for medical writing and illustration at the Medical Society of London on 2 March 1994. The winners were selected by the Executive Committee of the Medical Writers Group of the Society of Authors.

The £1,000 prize in the Atlas category was shared between **Dr Clifford M Lawrence** and **Dr Neil H Cox** for *Physical signs in dermatology,* published by Mosby Year Book in Europe.

The £1,000 prize in the Textbook category was awarded to **Professor R J Trent** for *Molecular medicine,* published by Churchill Livingstone.

The £1,000 prize in the Illustrated Text Category was shared between **Professor A R Mundy** and **Philip Wilson** for *Urodynamic and reconstructive surgery of the lower urinary tract,* published by Churchill Livingstone.