Molecular mechanisms of hepatic injury and repair

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ABSTRACT

In this thesis I examined molecular mechanisms involved in acute and chronic liver injury, and also studied basic pathways mediating tumour promotion.

Acute hepatic failure secondary to paracetamol poisoning is associated with high mortality. C-jun (NH2) terminal kinase (JNK) is a member of the mitogen activated protein kinase family and is a key intracellular signaling molecule involved in the control of cell fate. Paracetamol induced hepatic JNK activation in both human and murine paracetamol hepatotoxicity, and in a murine model preceded the onset of hepatocyte death. JNK inhibition *in vivo* (using two JNK inhibitors with different mechanisms of action) markedly reduced mortality in murine paracetamol hepatotoxicity. In addition, delayed administration of JNK inhibitor was more effective than N-acetylcysteine following paracetamol poisoning in mice. JNK inhibition was not protective in acute carbon tetrachloride or anti-Fas antibody mediated hepatic injury, suggesting specificity for the role of JNK in paracetamol hepatotoxicity. Furthermore, disruption of the *JNK1* or *JNK2* genes did not protect against paracetamol-induced hepatic damage. Pharmacological JNK inhibition had no effect on paracetamol metabolism, but markedly inhibited hepatic TNF-α production following paracetamol poisoning. These data demonstrate a central role for JNK in the pathogenesis of paracetamol induced liver failure, thereby identifying JNK as an important therapeutic target in the treatment of paracetamol hepatotoxicity.

Liver fibrosis with loss of tissue architecture and subsequent hepatic failure represents a massive healthcare burden worldwide. Expression of Galectin-3 (a β-galactoside binding animal lectin) is upregulated in established human fibrotic liver disease, during the development of experimental liver fibrosis and is temporally and spatially related to the induction and resolution of experimental hepatic fibrosis. Disruption of the gene encoding Galectin-3 blocks transdifferentiation of precursors to myofibroblasts *in vitro* and *in vivo*, markedly attenuating hepatic scarring in a murine model of liver fibrosis. Inhibition of Galectin-3 expression by siRNA in primary murine and human hepatic
stellate cells significantly reduced myofibroblast activation and procollagen(I) expression. The reduction in hepatic fibrosis observed in the \textit{Galectin-3^{-}} mouse occurred despite equivalent liver injury and inflammation, and similar tissue expression of TGF-\(\beta\). TGF-\(\beta\) failed to transactivate \textit{Galectin-3^{-}} hepatic stellate cells, in contrast with wild type hepatic stellate cells. However TGF-\(\beta\) stimulated signaling via Smad-2 and 3 was equivalent in both \textit{Galectin-3^{-}} and wild type hepatic stellate cells indicating that Galectin-3 is required for TGF-\(\beta\) mediated myofibroblast activation and matrix production. This supports a novel and important mechanistic role for Galectin-3 in the regulation of myofibroblast activation and consequent liver fibrosis. Finally, \textit{in vivo} siRNA knockdown of Galectin-3 inhibited myofibroblast activation following hepatic injury and may therefore provide a novel therapeutic approach to the prevention and treatment of liver fibrosis.

CD98hc (a ligand for Galectin-3) constitutively and specifically associates with \(\beta_1\) integrins and is highly expressed on the surface of human tumour cells irrespective of the tissue of origin. CD98hc promotes both anchorage- and serum-independent growth. Using chimeras of CD98hc and the type II membrane protein CD69 demonstrated that the transmembrane domain of CD98hc is necessary and sufficient for integrin association in cells. Furthermore, CD98hc/\(\beta_1\) integrin association is required for focal adhesion kinase-dependent phosphoinositol 3-hydroxykinase activation and cellular transformation. Amino acids 82-87 in the putative cytoplasmic/transmembrane region appear to be critical for the oncogenic potential of CD98hc and provide a novel mechanism for tumour promotion by integrins.
ACKNOWLEDGEMENTS

I am indebted to my supervisors, Professor Tariq Sethi and Dr Ken Simpson, for excellent guidance and support throughout my PhD; to Dr Alison MacKinnon for helping me learn the practicalities of laboratory work and for her invaluable help and advice; Professor John Iredale, who was a collaborator on my Fellowship, and an excellent “long-distance supervisor” in Southampton, now in Edinburgh. Finally, a very large thankyou to my wife Kirsty for her unerring support and understanding throughout my PhD studies.

DECLARATION

This thesis has been composed by myself and represents my own work. All of the experiments described herein were performed by myself with the exception of the CD98 colony assay work and CD98 immunofluorescence (chapter 4) which was done in collaboration with Dr Elizabeth Collis.
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<td>ALF</td>
<td>acute liver failure</td>
</tr>
<tr>
<td>ALT</td>
<td>alanine aminotransferase</td>
</tr>
<tr>
<td>α-SMA</td>
<td>alpha smooth muscle actin</td>
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<tr>
<td>ANOVA</td>
<td>one-way analysis of variance</td>
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<tr>
<td>BMDM</td>
<td>bone marrow derived macrophage</td>
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<tr>
<td>CCL₄</td>
<td>carbon tetrachloride</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid</td>
</tr>
<tr>
<td>CHO</td>
<td>chinese hamster ovary</td>
</tr>
<tr>
<td>DMEM</td>
<td>dulbecco's modified eagle's medium</td>
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<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
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<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>FRNK</td>
<td>focal adhesion kinase related non-kinase</td>
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<tr>
<td>HSC</td>
<td>hepatic stellate cell</td>
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<tr>
<td>IP₄</td>
<td>inositol 1,3,4,5-tetrakisphosphate</td>
</tr>
<tr>
<td>IP</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun (NH2) terminal kinase</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
</tr>
<tr>
<td>OLT</td>
<td>Orthotopic liver transplantation</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-hydroxykinase</td>
</tr>
<tr>
<td>PIP₃</td>
<td>phosphatidylinositol 3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PSR</td>
<td>picrosirius red</td>
</tr>
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<td>ROS</td>
<td>reactive oxygen species</td>
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CHAPTER 1

INTRODUCTION

The liver performs a myriad of vital functions to help maintain normal homeostasis. It has major biochemical roles in carbohydrate (gluconeogenesis, glycogenolysis and glycogenesis), lipid (cholesterol and triglyceride synthesis) and protein metabolism. The liver produces coagulation factors I (fibrinogen), II (prothrombin), V, VII, IX, X and XI, as well as protein C, protein S and antithrombin, and synthesises albumin, the major protein in blood. Bile components (required for dissolution and absorption of fats) are modified and/or synthesised in the liver, and the liver is also where most drugs and toxins, including alcohol, are metabolised. Furthermore, as well as biochemical functions, the liver has an important immunoregulatory role and constitutes a major component of the reticuloendothelial system. Therefore, when patients develop hepatic failure and many of the important functions listed above are disabled or lost, it is unsurprising that the morbidity and mortality rates associated with liver failure are substantial.

Acute liver failure

Acute liver failure (ALF) encompasses a number of conditions whose common theme is severe injury and massive necrosis of hepatocytes. This critical reduction in hepatocyte function triggers a multiorgan response, and mortality is high. Fulminant hepatic failure is defined as a syndrome in which hepatic encephalopathy, characterised by mental changes progressing from confusion to stupor and coma, results from sudden severe impairment of hepatic function. The syndrome is defined further as occurring within 8 weeks of onset of the precipitating illness, in the absence of pre-existing liver disease, to distinguish it from those instances in which hepatic encephalopathy represents a deterioration in chronic liver disease. Paracetamol (acetaminophen) is the commonest cause of ALF in the UK and USA, either following intentional overdose or inadvertent consumption of excessive amounts (O'Grady, 2005; Larson et al., 2005). Liver transplantation is still the only effective treatment for severe cases but is a limited resource (Simpson and Garden,
Therefore effective alternative treatments for paracetamol induced acute liver failure are urgently required.

Until recently toxin-induced hepatocyte injury was considered a passive process regulated predominantly by the dose of the injurious agent and its subsequent cellular metabolism. However, recent investigations have altered this concept and suggest that active intracellular signaling plays a crucial role in this process, with complex interplay between the pathways of toxin metabolism and the host inflammatory response (Jones and Czaja, 1998; Kaplowitz, 2004). The mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases that are evolutionary conserved signal transducing enzymes unique to eukaryotes (Johnson and Lapadat, 2002; Chang and Karin, 2001). The c-jun (NH2) terminal kinases (JNKs) are one subgroup of the MAPKs (Davis, 2000; Weston and Davis, 2002). JNK controls many basic mammalian physiological processes and phosphorylates specific subunits (c-Jun, JunB, JunD, and ATF-2) of the AP-1 transcription factor (Smeal et al., 1991; Pulverer et al., 1991; Ip and Davis, 1998) activating genes that regulate diverse cellular functions including cell proliferation (Sabapathy et al., 2004), survival (Xia et al., 1995; Shaulian et al., 2000) and death (Tournier et al., 2000; Varfolomeev and Ashkenazi, 2004). Our current understanding of the JNK pathway is summarised schematically in Figure 1.
The JNK pathway
Figure 1: Our current understanding of the JNK pathway.

CST website: http://www.cellsignal.com/category.asp?catalog_name=CellSignal&category_name=MAPK+Signaling
Many studies have highlighted the importance of the JNK pathway in liver pathophysiology. Although the regulation of cell death by JNK is contextual, recent in vitro data (Wang Y et al., 2004; Liu et al., 2002b; Schwabe et al., 2004; Graf et al., 2002) and in vivo studies (Kamata et al., 2005, Streetz et al., 2001; Uehara et al., 2005) utilising diverse mechanisms of hepatic injury including immune-mediated damage (Concanavalin A, TNF-α), bile acids and ischaemia / re-perfusion injury strongly support a role for JNK in the control of cell fate within the liver. Therefore, one of the aims of this thesis was to investigate whether JNK represents a functionally important pathway in the pathogenesis of paracetamol induced liver failure.

**Chronic liver failure**

Chronic liver failure arises in the context of extensive fibrosis of the liver and its end stage, cirrhosis, represents a massive healthcare burden worldwide. Liver cirrhosis is increasing in incidence globally: In the West the main causes are chronic hepatitis C infection, alcohol, and non-alcoholic steatohepatitis (NASH), whereas viral hepatitis (hepatitis B and C) predominate as the major causes of liver fibrosis in the middle East and Asia.

In the UK cirrhosis mortality rates increased steeply during the 1990s. Between the periods 1987-1991, and 1997-2001, cirrhosis mortality in men in Scotland more than doubled (104% increase) and in England and Wales rose by over two-thirds (69%). Mortality in women increased by almost half (46% in Scotland and 44% in England and Wales). Cirrhosis mortality rates in Scotland are now one of the highest in western Europe, in 2002 being 45.2 per 100,000 in men and 19.9 in women (Leon and McCambridge, 2006). Furthermore, changing patterns of alcohol consumption in the West and the increasing incidence of the metabolic syndrome mean that advances in preventing and treating viral hepatitis may be offset by an increasing burden of fibrosis and cirrhosis related to alcohol and non-alcoholic steatohepatitis (NASH).
Currently our therapeutic repertoire for the treatment of liver fibrosis and cirrhosis is severely limited. Broadly, treatment falls into two categories. Removal of the underlying injurious stimulus (where possible) such as viral eradication in Hepatitis B and C mediated liver disease, and removal of the terminally damaged organ as in liver transplantation (OLT) (Neuberger, 2000). OLT can be a highly successful treatment with an average five year survival rate of 75%. However transplantation has several disadvantages, including increasingly limited donor liver availability and the commitment of recipients to lifelong potentially toxic immunosuppression; in addition, patients may be excluded from transplantation as a result of psychiatric or medical comorbidities. Furthermore, in the specific case of hepatitis C induced cirrhosis (now the commonest indication for liver transplantation in the US), liver transplantation is far from curative as recurrence of hepatitis C in the graft is universal with uncertain graft and patient outcomes.

Liver fibrosis is an excellent example of the wound-healing response to iterative injury, and is the accumulation of extracellular matrix proteins and scarring which follows acute or chronic liver injury (Friedman, 2003). Cirrhosis, which is the end-point of the repetitive injury and progressive fibrosis continuum, is characterized by septum formation and rings of scar surrounding nodules of regenerating hepatocytes (Friedman, 2004). In the vast majority of cases hepatic fibrosis requires years or decades to become clinically apparent, but exceptions in which cirrhosis develops over a much shorter time-frame (in some cases months) include drug-induced liver disease, paediatric liver disease (e.g. biliary atresia), and viral hepatitis associated with immunosuppression after liver transplantation.

Following acute liver injury (for example viral hepatitis or following acute paracetamol injury), parenchymal cells (hepatocytes) regenerate and replace the necrotic or apoptotic cells. This repair process is associated with an inflammatory infiltrate and a limited deposition of extracellular matrix. In chronic liver injury hepatocyte number and function decreases, with distortion of the architecture of the liver and deposition of abundant extracellular matrix, including fibrillar collagens (Bataller and Brenner, 2005) (Figure 2).
Figure 2

Changes in the hepatic architecture (A) associated with advanced hepatic fibrosis (B)
**Figure 2:** Following chronic liver injury, inflammatory cells infiltrate the hepatic parenchyma. Some hepatocytes undergo apoptosis, and Kupffer cells activate, releasing fibrogenic mediators. HSCs proliferate and undergo a dramatic phenotypical activation, secreting large amounts of extracellular matrix proteins. Sinusoidal endothelial cells lose their fenestrations, and the tonic contraction of HSCs causes increased resistance to blood flow in the hepatic sinusoid. Bataller and Brenner, J. Clin. Invest. 2005;115:209-218.
Liver fibrosis is associated with major alterations in both the amount and composition of extracellular matrix (Benyon and Iredale, 2000). In advanced stages, the liver contains approximately six times more extracellular matrix than normal, including collagens (I, III, and IV), undulin, elastin, laminin, fibronectin, hyaluronan and proteoglycans. Furthermore, the distribution of matrix depends on the origin of the liver injury. In chronic cholestatic disorders and chronic viral hepatitis, the fibrotic tissue is initially located around portal tracts, while in alcohol-induced liver disease, it locates in pericentral and perisinusoidal areas (Pinzani, 1999). With the advance of chronic liver disease there is a progression from collagen bands to bridging fibrosis to frank cirrhosis.

Central to fibrogenesis and the scarring of organs is the activation of tissue fibroblasts into extracellular matrix-secreting myofibroblasts. Within the liver the main effector cells of fibrosis are the hepatic stellate cells (HSC - formerly known as lipocytes, Ito cells, or perisinusoidal cells) (Friedman et al, 1985). Classically, quiescent HSC become activated to a contractile, myofibroblast-like matrix-secreting phenotype. Figure 3 illustrates the major phenotypic changes seen following activation of HSC.
Figure 3

Hepatic stellate cell activation

Quiescent HSC

Liver injury

Activated HSC

Phenotypic changes following HSC activation:

↑ Proliferation
↑ Contractility
↑ Fibrogenesis
↑ ECM degradation
↑ HSC chemotaxis
Loss of retinoid
Figure 3: The major phenotypic changes seen after activation of hepatic stellate cells.
Activated HSC secrete fibrillar (or scarring) collagens, resulting in the deposition of fibrotic matrix, and can express tissue inhibitors of metalloproteinases (TIMPs) with the result that matrix degrading metalloproteinase activity is inhibited. This alters the balance of matrix secretion and degradation to favour accumulation. In this respect the activated stellate cell/myofibroblast can be considered as a final common cellular mediator of fibrosis. Interestingly, there is increasing evidence that liver myofibroblasts may derive from local sources other than HSC, and also from sites distant to the liver (Kruglov et al., 2006, Forbes et al., 2004, Russo et al., 2006).

The isolation of HSCs from animal and human livers has become increasingly refined (Otto and Veech, 1980, Friedman et al., 1992) and culture of HSCs on tissue culture plastic is now widely accepted as an in vitro model system for the study of HSC activation in vivo (Rockey et al., 1992). This has helped identify a number of pro-fibrotic mediators. With the development of genetically modified animals and their utilisation in models of liver fibrosis many of the previous in vitro studies have been corroborated in vivo and have aided further examination of the molecular mechanisms that drive liver injury, inflammation and fibrosis (Bataller and Brenner, 2005; Constandinou et al., 2005). Data presented within this thesis (Chapter 3) has identified Galectin-3 as a potential profibrotic mediator.

Galectin-3 (previous names Mac-2, L-29, CBP-35 or εBP for IgE-binding protein) is a β-galactoside binding animal lectin of approximately 30 kDa and is the sole member of the chimera-type family of galectins (Ho and Springer, 1982). It is abundantly present in the epithelia of several organs, as well as various inflammatory cells including activated macrophages, neutrophils, mast cells and T lymphocytes (Hughes, 1997). Although Galectin-3 is predominantly located in the cytoplasm, it is also detectable in the nucleus, on the cell surface and can be secreted into the extracellular environment, underlining the duality of function of this molecule both intracellularly and extracellularly.

Galectin-3 is found in solution as a monomer and is composed of two functional domains: a carboxyl-terminal domain that contains the carbohydrate-binding region and an amino-terminal domain consisting primarily of tandem repeats of nine amino acids to cross-link
carbohydrate and non-carbohydrate ligands (Jia and Wang, 1988; Cherayil et al., 1989; Liu, 1990; Hughes 1994; Birdsall et al., 2001; Krzeslak and Lipinska, 2004). The N-terminal domain also determines the secretion of Galectin-3 by a novel, non-classical pathway circumventing the endoplasmic reticulum–Golgi complex (Mehul and Hughes, 1997; Menon and Hughes, 1999). Increased Galectin-3 expression has been noted in tissue fibrosis (Kasper and Hughes, 1996; Hsu et al., 1999; Wang L et al., 2000), and in vitro exogenous Galectin-3 stimulates myofibroblast proliferation (Sasaki et al., 1999; Maeda et al., 2003). However, the relevance of these observations to the mechanistic role of Galectin-3 in the pathogenesis of tissue fibrosis in vivo had not been examined.

### Ligands for Galectin-3

Table 1 illustrates the growing list of ligands for Galectin-3 found both inside and outside the cell. These putative ligands have been identified using a variety of techniques including affinity column pull-down and yeast 2-hybrid screens. The functional significance of a number of the interactions remains to be elucidated. It is noteworthy that almost all of the listed intracellular ligands interact with galectin-3 via protein-protein rather than lectin-glycoconjugate interactions. One of the few exceptions is the cytokeratins. Galectin-3 has been shown to bind to cytokeratins in a fashion that is dependent on the presence of GalNAc residues on the latter (Goletz et al., 1997). However it remains to be shown that such carbohydrate-bearing ligands play a role in the intracellular activities of galectins.
<table>
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<tr>
<th>Ligand</th>
<th>Source/cells</th>
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<td><strong>Extracellular matrix proteins</strong></td>
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<tr>
<td>Laminin</td>
<td>EHS, macrophage, placenta</td>
<td>Woo et al., 1990</td>
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<td></td>
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<td>Sato et al., 1992</td>
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<tr>
<td></td>
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<td>Ochieng et al., 1995</td>
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<td>Fibronectin</td>
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<td>Tenascin</td>
<td>Brain</td>
<td>Probstmeier et al., 1995</td>
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<td>M2BP</td>
<td>Brain</td>
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<td><strong>Membrane proteins</strong></td>
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<td>Integrins:</td>
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<td>αM/β2(CD11b/18)</td>
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<td>α1/β1</td>
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<td></td>
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<td>Zhu et al., 2001</td>
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Adapted from Krzeslak et al., 2004.
Molecular mechanisms of cellular transformation

Within tissues acute inflammation can evolve into chronic inflammation with the formation of fibrous scar tissue, and a resultant increased predisposition to malignant transformation. Many cancers show abnormalities of integrin function as a result of transformation by oncogenes (Zou et al., 2002). More importantly, the growth of several tumours depends on β1 integrin function (Weaver et al., 1997). As stated above, Galectin-3 has been identified as a ligand for the transmembrane protein CD98 (an early T-cell activation antigen) (Dong and Hughes, 1997) (see Table 1). CD98 has been identified as a central activator of integrin function (Fenczik et al., 1997). Galectin-3 was further shown to be an endogenous cross-linker of the CD98 antigen, resulting in the activation of integrin mediated adhesion suggesting that Galectin-3 could promote CD98 dimerization and, indirectly, integrin activation (Hughes, 2001). Previous work demonstrated that cross-linking CD98 (with the 4F2 monoclonal antibody) promoted integrin-like signaling and anchorage-independent growth (Rintoul et al., 2002). However, the mechanism by which CD98hc associates with and regulates integrin function and what role this plays in transformation is unclear. The third aim of this thesis was to investigate the mechanism by which overexpression of CD98hc (a ligand for Galectin-3) leads to cellular transformation, in particular assessing the relationship between transformation, PI3K activation, and β1 integrins.
CHAPTER 2

Critical role of c-jun (NH2) terminal kinase in paracetamol-induced acute liver failure

2.1 Abstract

Acute hepatic failure secondary to paracetamol poisoning is associated with high mortality. C-jun (NH2) terminal kinase (JNK) is a member of the mitogen activated protein kinase family and is a key intracellular signaling molecule involved in the control of cell fate. Using a previously developed mouse model of paracetamol poisoning, we examined in detail the role of JNK in paracetamol induced acute liver failure. We demonstrate that paracetamol induced hepatic JNK activation in both human and murine paracetamol hepatotoxicity, and in our murine model preceded the onset of hepatocyte death. JNK inhibition \textit{in vivo} (using two JNK inhibitors with different mechanisms of action) markedly reduced mortality in murine paracetamol hepatotoxicity, with a significant reduction in hepatic necrosis and apoptosis. In addition, delayed administration of JNK inhibitor was more effective than N-acetylcysteine following paracetamol poisoning in mice. JNK inhibition was not protective in acute carbon tetrachloride or anti-Fas antibody mediated hepatic injury, suggesting specificity for the role of JNK in paracetamol hepatotoxicity. Furthermore, disruption of the \textit{JNK1} or \textit{JNK2} genes did not protect against paracetamol-induced hepatic damage. Pharmacological JNK inhibition had no effect on paracetamol metabolism, but markedly inhibited hepatic TNF-\(\alpha\) production following paracetamol poisoning. These data demonstrate a central role for JNK in the pathogenesis of paracetamol induced liver failure, thereby identifying JNK as an important therapeutic target in the treatment of paracetamol hepatotoxicity.
2.2 Introduction

The metabolic activation of paracetamol to N-acetyl-p-quinoneimine (NAPQI) and subsequent conjugation with glutathione is well characterized (Nelson et al., 2002). However, the pathways linking paracetamol metabolism with hepatic necrosis remain elusive. Recent investigations have expanded our understanding of the complex interplay between the pathways of toxin metabolism, intracellular signalling and the host inflammatory response (Jones and Czaja, 1998; Kaplowitz, 2004). Inflammatory cells play a permissive role in hepatic necrosis induced by paracetamol and early response cytokines and chemokines modulate both the injury and repair processes (Ishida et al., 2002; Hogaboam et al., 1999a). Therefore further study of inflammatory cell biology and the intracellular signaling pathways which regulate the injurious response in paracetamol toxicity may yield targeted new treatments for patients with paracetamol poisoning.

Many studies have highlighted the importance of the JNK pathway in liver pathophysiology. Although the regulation of cell death by JNK is contextual, recent in vitro data (Wang Y et al., 2004; Liu et al., 2002; Schwabe et al., 2004; Graf et al., 2002) and in vivo studies (Kamata et al., 2005, Streetz et al., 2001; Uehara et al., 2005) utilising diverse mechanisms of hepatic injury including immune-mediated damage (Concanavalin A, TNF-α), bile acids and ischaemia / re-perfusion injury strongly support a role for JNK in the control of cell fate within the liver. Therefore, one of the aims of this thesis was to investigate whether JNK represents a functionally important pathway in the pathogenesis of paracetamol induced liver failure.
2.3 Materials and Methods

**Human biopsy specimens and animal models**

Archival human liver samples were obtained from the Department of Pathology, University of Edinburgh. All animal procedures were undertaken using C57/BL6 mice with approved license from the Animal Scientific Procedures Division of the Home Office (London, UK) using mice aged 8-10 weeks old. Generation of \( JNK1^{-/-} \) and \( JNK2^{-/-} \) mice by gene-targeting technology (on a C57/BL6 background) has been described previously (Dong *et al.*, 1998). As control, age and sex-matched wild-type (WT) littermates were used. After overnight fast, mice were injected IP with 350 mg/kg of paracetamol dissolved in sterile PBS and warmed to 42\(^\circ\)C as described (Hogaboam *et al.*, 2000), CCL\(_4\) in a ratio of 1:3 with olive oil at a dose of 1ul/g body weight as previously described (Henderson *et al.*, 2006) or anti-Fas antibody (0.5 µg/g body weight) (BD Pharmingen, UK). Two JNK inhibitors were used in this study: SP600125 (anthra[1,9-cd]pyrazol-6(2H)-one) purchased from Calbiochem (San Diego, CA) and D-JNKI1 inhibitor [c-Jun N-terminal Kinase Peptide Inhibitor 1, D-stereoisomer] purchased from Axxora Ltd. (Nottingham, UK). Vehicle control for SP600125 was 40% polyethylene glycol (PEG) (Sigma, UK) in PBS. Control for D-JNKI1 was D-TAT control peptide (Axxora Ltd., Nottingham, UK) in PBS. In experiments utilising delayed JNK inhibition, pharmacological inhibitors or appropriate controls were administered IP 5 hours after paracetamol injection.

**Analysis of liver injury**

After IP injection, groups of mice were scored as follows by a blinded observer for signs of systemic illness: clinically well (0 points), presence of piloerection (1 point), with additional hunched posture (2 points), and lack of spontaneous movement (3 points) at 24 hours post injection. Serum was stored at -80\(^\circ\)C until use for determination of alanine aminotransferase levels by an automated enzyme assay (Olympus 20700 analyser). Two lobes from each liver were fixed in 4% paraformaldehyde before routine histological processing. Histological grading of hepatic necrosis was performed by two blinded
observers using H&E stained sections as follows: less than 30% of the total area necrotic (1 point); 30-60% of the total area necrotic (2 points); greater than 60% of the total area necrotic (3 points). Apoptotic counting: ten random fields were counted independently by two blinded observers. Whole liver homogenates were prepared and the Cell Death Detection ELISA (quantitative detection of histone-associated DNA fragments in mono- and oligonucleosomes) used as per manufacturer’s instructions (ROCHE, UK).

**Protein analysis**

Paraffin-embedded sections were deparaffinised and subjected to microwave antigen retrieval in citrate buffer. The primary antibodies were: rabbit polyclonal anti-phospho-JNK (Biosource, Belgium) and anti-paracetamol protein-adduct antibody (HyCult biotechnology, Netherlands). Species appropriate isotype control antibodies were also used for each experiment. Western blot analysis was undertaken by using the following primary antibodies: rabbit polyclonal anti-phospho-JNK [pTpY183/185] (Biosource, Belgium), rabbit polyclonal anti-phospho p38 antibody [pTpY180/182] (Biosource, Belgium) and rabbit polyclonal anti-β-actin antibody (Sigma, UK). JNK activity was measured as described previously (MacKinnon and Sethi, 2003). Liver was homogenized in PBS with protease inhibitor cocktail (Roche, UK) and immunoreactive tumour necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ) measured in the supernatants by ELISA according to manufacturer’s instructions (R & D systems, UK).

**Statistical analysis**

Results are presented as means ± S.E.M. Significance of the differences between means was assessed using one-way analysis of variance (ANOVA) or two-tailed Student's *t* test. Values of *P* < 0.05 were considered significant. Unless stated otherwise, studies were performed on three to six independent occasions using 6-8 mice per group.
2.4 Results

Paracetamol induces hepatic JNK activation in human and murine liver and precedes the onset of hepatocyte death.

To determine whether JNK activity is increased during paracetamol induced liver injury we examined phospho-JNK expression. In normal human liver constitutive JNK phosphorylation occurs in areas surrounding the central veins (Fig. 1a). Cytoplasmic and dense nuclear staining was observed within hepatocytes. Following paracetamol induced ALF there was a marked increase in hepatocyte cytoplasmic and nuclear phospho-JNK staining compared with control normal liver (Fig. 1b) (n=8 cases in each group). We also examined JNK activation in a murine model of paracetamol-induced hepatic injury. Phospho-JNK expression was negligible in control mouse liver (Fig. 1c). Similar to human cases, paracetamol injured mouse liver demonstrated marked upregulation of phospho-JNK in the hepatocyte nuclei and cytoplasm in the areas surrounding the central veins (Fig. 1d). The time course of JNK activation following paracetamol injury was examined: hepatic JNK activity dramatically increased following paracetamol administration with peak activity at 2 hours post-injection (Fig.1 e and inset). JNK activity then returned towards basal levels by 24 hours. Comparison with the time course of biochemical liver injury (determined by serum alanine aminotransferase, ALT) showed peak ALT 5 hours following paracetamol injection (Fig. 1f.).
Figure 1

Normal Paracetamol

Human

Paracetamol

Control Mouse

Paracetamol

P-JNK

P-JNK

Percentage increase

0 1 2 5 24

Hours

0 1 2 5 24

Hours

P-c-jun

<image description>

<image description>
Figure 1

![Bar graph showing ALT (IU/L) levels at different hours.](image)

- **ALT (IU/L)**
- **Hours**: 0, 1, 2, 5, 24
- **Values**: 0, 15000, 10000, 5000

**Significance**: ****
Figure 1

Paracetamol induces hepatic JNK activation in both human and murine paracetamol-induced liver injury, and precedes the onset of hepatocyte death.

(a) Phospho-JNK expression in normal human liver and (b) in human paracetamol-induced liver injury (n=8 cases in each group). Scale bar 400 µm. (c) Phospho-JNK expression in mouse liver 2 hours after IP administration of control alone. (d) Phospho-JNK expression in mouse liver 2 hours after treatment with paracetamol (350 mg/kg IP). Scale bar 200 µm. (e) Timecourse of hepatic JNK activation in mouse liver following paracetamol-induced hepatic injury (350 mg/kg IP). Inset: representative phosphorimage of hepatic JNK activity in whole liver homogenates from paracetamol treated mice. (f) Timecourse of serum alanine aminotransferase (ALT) release in mice following paracetamol-induced liver injury (350 mg/kg IP) (**p<0.01).
Pharmacological inhibition of JNK activity in vivo markedly reduces murine mortality and liver injury.

To assess the pathogenic significance of the observed increase in JNK activity we used two pharmacological inhibitors of JNK which differ in their mechanisms of action. SP600125 is a small molecule reversible ATP-competitive inhibitor (Bennett et al., 2001; Han et al., 2001) whereas D-JNKI1 is a peptide inhibitor which inhibits the interaction of JNK with its substrates (Borsello et al., 2003). Immunohistochemistry for hepatic phospho-JNK demonstrated potent inhibition of JNK phosphorylation by SP600125 at 2 hours post paracetamol treatment compared with control (Fig. 2a,b) and was confirmed by radioactive kinase assay (Fig. 2c) and western blotting for phospho-JNK (Fig. 2d). Importantly, pre-treatment with SP600125 did not inhibit phosphorylation of the other major stress activated protein kinase in the liver, p-38 (Fig. 2d). To determine the potential clinical significance of JNK activation following paracetamol poisoning the JNK inhibitors (or control) were injected one hour prior to paracetamol administration, and mortality was assessed over 72 hours. Inhibition of JNK activity with either SP600125 or D-JNKI1 dramatically reduced mortality compared with vehicle control (Fig. 2e). In addition sickness scores were assessed at 24 hours and the SP600125 and D-JNKI1 inhibitor groups exhibited significantly lower sickness scores compared with the vehicle control group (Fig. 2f).
Figure 2

Control + paracetamol  SP600125 + paracetamol

P-JNK

P-c-jun

0 2 24 0 2 24 Hours
Control SP600125

P-JNK
P-p38
β-actin

- + - + - + SP600125
0 2 24 Hours
Figure 2

e
![Bar graph showing percentage mortality across different conditions.](image)

f

![Plot showing sickness score at 24 hours across different conditions.](image)
Figure 2

Pharmacological inhibition of JNK activity \textit{in vivo} markedly reduces mortality in a mouse model of paracetamol-induced acute liver failure.

Mice were injected IP with vehicle control or SP600125 (30mg/kg) one hour prior to IP injection with paracetamol solution (350 mg/kg). (a) Hepatic phospho-JNK expression in the vehicle control group 2 hours after paracetamol administration. (b) Hepatic phospho-JNK expression in the SP600125 group 2 hours after paracetamol administration. (c) Timecourse of hepatic JNK activity in mice injected IP with vehicle control or SP600125 (30mg/kg) one hour prior to paracetamol administration (350 mg/kg). (d) Timecourse of hepatic phospho-JNK and phospho-p38 in mice injected IP with vehicle control or SP600125 (30mg/kg) one hour prior to paracetamol administration (350 mg/kg). (e) Mortality at 72 hours in mice injected IP with vehicle control, SP600125 (30mg/kg) or D-JNKI1 (30µg/mouse) one hour prior to paracetamol administration (450 mg/kg) \(n=10\) mice in each group). (f) Sickness scores at 24 hours in mice injected IP with vehicle control, SP600125 (30mg/kg) or D-JNKI1 (30µg/mouse) one hour prior to paracetamol administration (350 mg/kg) \(n=10\) mice in each group). Each point represents an individual mouse.
To further evaluate the striking survival benefit of JNK inhibition following paracetamol administration, liver injury was assessed following JNK inhibition. SP600125 or D-JNKI1 dramatically reduced histological liver injury and hepatic necrosis scores compared with vehicle control (Fig. 3a,b). Furthermore, hepatocyte necrosis, as assessed by serum ALT release, was also reduced in the JNK inhibitor groups compared with vehicle control (Fig. 3c). Hepatocyte apoptosis may occur during paracetamol toxicity in human and murine liver (McGregor et al., 2003). Apoptotic cells were clearly visible in the control group at 5 hours post paracetamol administration, however apoptotic cells were less abundant in the JNK inhibitor groups at this time point (Fig. 3d). Counting of morphologically apoptotic cells confirmed this finding with significant reduction in the number of apoptotic hepatocytes in the inhibitor groups compared with control, 5 hours post paracetamol (Fig. 3e). Further assessment of apoptosis by cell death ELISA (quantitative detection of histone-associated DNA fragments in mono- and oligonucleosomes) utilising whole liver homogenates also demonstrated a significant reduction in apoptosis in the JNK inhibitor groups at 1, 2 and 5 hours post paracetamol treatment compared with control (Fig. 3f, p < 0.01).
Figure 3

a

Paracetamol

Control SP600125 D-JNKI1

b

Hepatic necrosis score

Control SP60 D-JNKI1

***
Figure 3

c

![Bar chart showing ALT (IU/L) levels for Control, SP60, and D-JNKI1]

Control  SP60  D-JNKI1

ALT (IU/L)

7500
5000
2500

0

* 

---

d

![Images of tissue samples labeled Control, SP600125, and D-JNKI1 with Paracetamol treatment]

Control  SP600125  D-JNKI1

Paracetamol

Arrows pointing to specific areas in the images.
Figure 3

**Apoptotic cells / HPF**

- Control
- SP600125
- D-JNKI1

**Optical Density**

- Control
- SP600125
- D-JNKI1

**Hours**

0 1 2 5 24

**Figure 3 continued...**
Figure 3

JNK inhibition *in vivo* reduces hepatic necrosis and apoptosis in paracetamol-induced acute liver failure.

Mice were injected IP with vehicle control, SP600125 (30mg/kg) or D-JNKI1 (30µg/mouse) one hour prior to paracetamol administration (350 mg/kg) (n=6 mice in each group). (a) Liver histology at 24 hours post paracetamol. Scale bar 200 µm. (b) Hepatic necrosis scores at 24 hours post paracetamol (**p<0.0001). (c) Serum alanine aminotransferase (ALT) release at 24 hours post paracetamol (*p<0.05). (d) Liver histology at high magnification (x200) 5 hours after paracetamol administration demonstrating apoptotic cells (arrowed) in the control vehicle treated group. (e) Timecourse of apoptotic cells/high power field (**p<0.01). (f) Timecourse of apoptosis assessed by cell death ELISA (quantitative detection of histone-associated DNA fragments in mono- and oligonucleosomes) from whole liver homogenates (**p<0.01).
Delayed administration of JNK inhibitor is more effective than N-acetylcysteine (NAC) in limiting liver injury.

An important clinical problem in the management of paracetamol poisoning is the group of patients who present 15 hours or more after the overdose when the antidote, NAC, is much less effective. We have previously used NAC in a mouse model of paracetamol toxicity (Hogaboam et al., 1999b) and demonstrated that beyond five hours NAC is no longer effective in preventing liver injury. Therefore we investigated whether delayed administration of JNK inhibitor is more effective than NAC when given after paracetamol overdose. Histological liver injury was less in the NAC and SP600125 groups (Fig 4a). Furthermore, SP600125 was significantly more effective than NAC in limiting liver injury following paracetamol poisoning when given 5 hours post overdose as assessed by hepatic necrosis scoring (Fig 4b) and serum ALT release (Fig 4c). However, JNK inhibition was no longer effective at 8 and 24 hours post paracetamol poisoning.
Figure 4

a

Paracetamol

Control NAC SP600125

b

Hepatic necrosis score

Control NAC SP60

*
Figure 4

ALT (IU/L)

Control  NAC  SP60

*
Figure 4
Delayed administration of JNK inhibitor is more effective than N-acetylcysteine (NAC) in limiting liver injury following paracetamol poisoning in mice.

Mice were treated with paracetamol (350 mg/kg IP) and 5 hours later administered control vehicle, NAC or SP600125 (30mg/kg) (n=6 mice in each group). (a) Liver histology at 24 hours post paracetamol. Scale bar 200 µm. (b) Hepatic necrosis scores at 24 hours post paracetamol (*p<0.05). (c) Serum alanine aminotransferase (ALT) release at 24 hours post paracetamol (*p<0.05).
JNK inhibition is not protective in acute carbon tetrachloride (CCL₄) or anti-Fas antibody mediated hepatic injury.

To determine whether JNK inhibition is protective in other forms of acute liver injury we examined JNK inhibition in acute CCL₄ and anti-Fas antibody induced hepatic damage. Similar to paracetamol, CCL₄ is a hepatotoxin metabolised in the liver by the cytochrome system leading to the release of toxic free radicals and oxidant mediated hepatic injury. Also CCL₄ injection increases JNK activity in whole liver *in vivo* (Mendelson *et al.*, 1996). To assess the effect of JNK inhibition in CCL₄ mediated liver injury, mice received CCL₄ (CCL₄ in a ratio of 1:3 with olive oil at a dose of 1ul/g body weight) one hour after treatment with control, SP600125 or D-JNKI1. Similar histological liver injury was observed in all groups (Fig. 5a) and there was no significant difference in hepatic necrosis scores (Fig. 5b) or biochemical liver injury at 24 hours (Fig. 5c). Further experiments were also undertaken using CCL₄ in a ratio of 1:3 with olive oil at a dose of 0.5ul/g body weight with no protection provided by JNK inhibition.

Previous studies have suggested that the Fas death pathway may play a role in the pathogenesis of paracetamol-induced liver injury (Zhang *et al.*, 2000; Liu *et al.*, 2004; Tagami *et al.*, 2003). Therefore we specifically examined the role of JNK inhibition in the anti-Fas antibody model of hepatic injury. Administration of anti-Fas (Jo-2) antibody directly ligates and activates the CD95 death receptor pathway resulting in massive hepatocyte apoptosis within hours (Ogasawara *et al.*, 1993; Galle *et al.*, 1995). Mice received anti-Fas antibody one hour after treatment with control, SP600125 or D-JNKI1. Similar histological liver injury was observed in all groups (Fig. 5d) and there was no significant difference in hepatic necrosis scores (Fig. 5e) or biochemical liver injury at 6 hours (Fig. 5f). Further experiments were also undertaken using anti-Fas antibody at a dose of 0.25 µg/g body weight with no protection provided by JNK inhibition (data not shown).
Figure 5

a

CCL4

Control  SP600125  D-JNKI1

b

Hepatic necrosis score

Control  SP60  D-JNKI1
Figure 5

c

![Bar chart showing ALT (IU/L) levels for Control, SP60, and D-JNK11 groups.](chart)

ALT (IU/L)

Control SP60 D-JNK11

0 2500 5000 7500 10000

d

![Images of liver sections labeled Control, SP600125, and D-JNK11 with anti-Fas antibody.](images)

Control SP600125 D-JNK11

Anti-Fas ab
Figure 5

(e) Hepatic necrosis score

(f) ALT (IU/L)
Figure 5

JNK inhibition is not protective in acute carbon tetrachloride (CCL₄) or anti-Fas antibody mediated hepatic injury.

CCL₄ model: Mice were administered CCL₄ in a ratio of 1:3 with olive oil at a dose of 1ul/g body weight one hour after treatment with control, SP600125 (30mg/kg) or D-JNKI1 (30µg/mouse) (n=6 mice in each group). (a) Liver histology at 24 hours post CCL₄ administration. Scale bar 200 µm. (b) Hepatic necrosis scores 24 hours post CCL₄ administration (p=NS). (c) Serum alanine aminotransferase (ALT) release at 24 hours post CCL₄ induced liver injury (p=NS).

Fas model: Mice were administered anti-Fas antibody (0.5 µg/g body weight) one hour after treatment with control, SP600125 (30mg/kg) or D-JNKI1 (30µg/mouse) (n=6 mice in each group). (d) Liver histology at 6 hours after anti-Fas antibody administration. Scale bar 200 µm. (e) Hepatic necrosis scores 6 hours after anti-Fas antibody administration (p=NS). (f) Serum alanine aminotransferase (ALT) release at 6 hours post anti-Fas antibody induced liver injury (p=NS).
Disruption of the *JNK1* or *JNK2* genes does not protect against paracetamol-induced liver injury.

The JNK protein kinases are encoded by three genes (Davis, 2000). The liver expresses two of the three known JNK genes – JNK 1 and JNK 2. To dissect in further detail the role of JNK1 and JNK2 in paracetamol-induced hepatic failure we studied *JNK1*\(^{-/-}\) and *JNK2*\(^{-/-}\) mice (*JNK1*\(^{+/-}\) double null mice are embryonic lethal). Liver histology showed no protection in the *JNK1*\(^{-/-}\) and *JNK2*\(^{-/-}\) mice compared with WT (Fig. 6a) and no significant difference in hepatic necrosis scores (Fig. 6b) or biochemical liver injury between groups (Fig. 6c).
Figure 6

a

Paracetamol

WT  JNK1<sup>−/−</sup>  JNK2<sup>−/−</sup>

b

Hepatic necrosis score

WT  JNK1<sup>−/−</sup>  JNK2<sup>−/−</sup>
Figure 6

ALT (IU/L)
Figure 6

Disruption of the *JNK1* or *JNK2* genes does not protect against paracetamol-induced liver injury.

Wild type, *JNK1*<sup>−/−</sup> or *JNK2*<sup>−/−</sup> mice were administered paracetamol (350 mg/kg IP) and livers and serum harvested at 24 hours (*n*=6 mice in each group). (a) Liver histology at 24 hours post paracetamol administration. Scale bar 200 µm. (b) Hepatic necrosis scores at 24 hours post paracetamol administration (*p*=NS). (c) Serum alanine aminotransferase (ALT) release at 24 hours post paracetamol-induced liver injury (*p*=NS).
JNK inhibition does not affect synthesis of paracetamol protein-adducts, but markedly inhibits TNF-α production in the liver following paracetamol-induced hepatic injury.

Ingestion of paracetamol results in metabolism to N-acetyl-p-quinoneimine (NAPQI) which is effectively detoxified by conjugation with glutathione. However, in paracetamol poisoning cytosolic and mitochondrial glutathione becomes deplete allowing covalent binding of NAPQI to hepatocellular proteins (paracetamol-protein adducts) (Nelson and Bruschi, 2002). Although the exact mechanisms mediating paracetamol hepatotoxicity remain elusive, recent data has highlighted the important regulatory role of the immune system and cytokine networks in determining outcome following paracetamol induced liver injury (Hogaboam et al., 1999a; Hogaboam et al., 2000; Hogaboam et al., 1999b; Liu et al., 2004; Bone-Larson et al., 2001). Therefore we examined whether JNK inhibition interferes with the metabolism of paracetamol by measuring paracetamol-protein adducts (James et al., 2003). No paracetamol-protein adducts were seen in normal mouse liver (Fig. 7a). However, paracetamol-protein adduct formation (visible in hepatocytes around the central veins) was similar in all groups 5 hours post-paracetamol (vehicle control, SP600125 and D-JNKI1 groups) (Fig. 7b-d).

We also explored in vivo which inflammatory cytokines may be modulated by JNK inhibition in our model of paracetamol induced liver injury. Previous studies have implicated interferon-γ (IFN-γ) in the pathogenesis of paracetamol-induced liver injury (Ishida et al., 2002; Liu et al., 2004). Hepatic IFN-γ levels were elevated compared to control in all groups at 2 hours post paracetamol administration, and remained elevated throughout 24 hours. No significant difference in hepatic IFN-γ levels was observed between the treatment groups at any of the time-points studied (Fig. 7e).

Tumor necrosis factor-α (TNF-α) is an inflammatory cytokine which may play a significant pathogenic role in paracetamol hepatotoxicity (Hogaboam et al., 2000; Ishida et al., 2004; Matsumaru et al., 2003), although conflicting data has been reported. In vitro studies show that TNF-α induces JNK activation in hepatocytes (Schwabe et al., 2006). In addition to the role of TNF-α in apoptotic hepatocyte death, perhaps more relevant to
paracetamol induced liver injury, the JNK/AP-1 signaling pathway can also mediate TNF-α induced necrotic hepatocyte death (Liu et al., 2002a). JNK activation can also induce TNF-α expression via AP-1 (Swantek et al., 1997, Kontoyiannis et al., 1999). Therefore, we sought to examine whether the protective effect of JNK inhibition in paracetamol hepatotoxicity is mediated by modulation of TNF-α expression. Hepatic TNF-α levels were significantly elevated in the vehicle control group at 2 and 5 hours post paracetamol compared with baseline (0 hours). However, there was significantly less hepatic TNF-α production in the JNK inhibitor groups at 2 and 5 hours post paracetamol compared with vehicle control (Fig. 7f).
Figure 7

**e**

![Bar graph showing IFN-γ levels](image)

- **Control**
- **SP600125**
- **D-JNKI1**

**f**

![Bar graph showing TNF-α levels](image)

- **Control**
- **SP600125**
- **D-JNKI1**

* Indicates significant changes.
Figure 7

JNK inhibition does not affect synthesis of paracetamol protein-adducts, but markedly inhibits TNF-α production in the liver following paracetamol-induced hepatic injury.

Mice were injected IP with control, SP600125 (30mg/kg) or D-JNKII (30µg/mouse) one hour prior to paracetamol administration (350 mg/kg) (n=6 mice in each group). (a) Anti-paracetamol protein-adduct staining in normal, untreated mouse liver. Scale bar 200 µm. (b-d) Anti-paracetamol protein-adduct formation at 5 hours post paracetamol administration. (e) Timecourse of hepatic IFN-γ production following paracetamol administration (p=NS). (f) Timecourse of hepatic TNF-α production following paracetamol administration (*p<0.05).
2.5 Discussion

We have shown a central role for JNK in the pathogenesis of paracetamol induced ALF, thereby identifying JNK as a potential therapeutic target in the treatment of paracetamol hepatotoxicity. Our data indicate: 1) Paracetamol-induced liver injury results in hepatic JNK activation in human and murine tissue which precedes the onset of hepatocyte death. 2) Pharmacological inhibition of JNK \textit{in vivo} markedly improved survival in a mouse model of paracetamol-induced ALF and decreased both hepatic necrosis and apoptosis. The protective effect of JNK inhibition was specific for paracetamol hepatotoxicity as the inhibitors were not protective in acute CCL$_4$ or anti-Fas antibody mediated hepatic injury. 3) Delayed administration of JNK inhibitor is more effective than N-acetylcysteine in limiting liver injury in mice. 4) Disruption of either the \textit{JNK1} or \textit{JNK2} genes was not protective. 5) Inhibition of JNK had no effect on paracetamol metabolism, but inhibited hepatic TNF-$\alpha$ production following paracetamol-induced ALF.

Paracetamol is the commonest cause of ALF in the UK and USA, causing significant morbidity and mortality in predominantly young people. Many patients present outside the therapeutic window for the commonly used antidote, N-acetylcysteine. Our data demonstrated that delayed administration of JNK inhibitor is more effective than NAC following paracetamol poisoning in mice. Although in clinical practice NAC can be administered up to 15 hours post-paracetamol overdose, we found limited efficacy at 5 hours post-paracetamol in the mouse model. This is in keeping with our previously published data (Hogaboam \textit{et al.}, 1999b) and relates to the truncated time course of liver injury in mice compared with humans. Metabolic activation of paracetamol and the histological liver injury produced in mice is similar to that observed in humans, however the variability in terms of the clinical context in which paracetamol overdose is taken and the genetic background of humans limits direct extrapolations of our results into treatment of patients. Despite these limitations, our data indicate further study of JNK activation in human cases of paracetamol poisoning may lead to the development of novel therapies and that pharmacological inhibition of JNK may be of particular clinical use in the group of patients with delayed presentation following paracetamol overdose.
Our data show prolonged hepatic activation of JNK following paracetamol overdose in both human and murine liver. Interestingly, in normal human liver phospho-JNK staining was observed in hepatocytes surrounding the central veins, the area in which paracetamol is preferentially activated by the hepatic cytochrome P450 system. Hepatic JNK activation may be consequent upon the oxidative stress produced during paracetamol metabolism. Reactive oxygen species (ROS) are widely recognised to induce increased and/or prolonged JNK activation, possibly due to inactivation of cellular phosphatases (Kamata et al., 2005). Although ROS alone can activate JNK, hepatic TNF-α expression is increased in paracetamol induced liver injury (Blazka et al., 1995; Blazka et al., 1996; Simpson et al, 2000). TNF-α is a potent inducer of both JNK and ROS. Therefore in the hepatic cytokine milieu induced by paracetamol, a positive amplification loop may exist whereby paracetamol induced ROS leads to JNK activation and TNF-α expression via AP-1 (Swantek et al., 1997; Kontoyiannis et al., 1999), resulting in further prolonged massive JNK activation in the liver via TNF receptor signaling (Fig. 8).

To investigate the potential pathogenic role of JNK activation in the development of paracetamol-induced hepatic necrosis, we employed two different JNK inhibitors with different mechanisms of action (SP600125 and D-JNKI1). Both inhibitors have been widely studied both in vivo and in vitro. Although the specificity of pharmacological inhibitors can be questioned, we found no reduction in the hepatic activation of another stress activated protein kinase, p38, following administration of JNK inhibitor and paracetamol. Furthermore, despite different mechanisms of action, both JNK inhibitors conferred significant survival benefit and profound protection against paracetamol induced hepatic necrosis.
Figure 8

Paracetamol

\[ \text{TNF-} \alpha \]

\[ \rightarrow \text{TNF-} \alpha \]

\[ \text{Cell membrane} \]

\[ \rightarrow \text{ROS} \rightarrow \text{JNK activation} \]

\[ \text{Nuclear membrane} \]

\[ \rightarrow \text{AP-1} \rightarrow \text{TNF-} \alpha \text{ production} \]

\[ \text{Hepatocyte necrosis} \]

\[ \text{Hepatocyte apoptosis} \]
Figure 8

Postulated positive amplification loop whereby paracetamol induced ROS in hepatocytes leads to JNK activation and TNF-α expression via AP-1, resulting in further prolonged massive JNK activation in the liver via TNF receptor signaling.
Several studies have shown the mechanistic importance of the hepatic cytokine network in paracetamol-induced liver injury. To investigate the mechanisms underlying the protective effect of JNK inhibition in paracetamol hepatotoxicity, we measured hepatic expression of IFN-γ and TNF-α. Both these cytokines are induced in the liver following paracetamol poisoning (Ishida et al., 2002; Liu et al., 2004; Blazka et al., 1995; Blazka et al., 1996; Simpson et al., 2000). Furthermore, IFN-γ knockout mice are resistant to paracetamol induced liver injury (Ishida et al., 2002). However, in our model JNK inhibition had no significant effect on hepatic IFN-γ. In contrast, JNK inhibition significantly reduced hepatic TNF-α expression. We and others have shown increased TNF-α expression in the peripheral circulation and liver following paracetamol poisoning (Hogaboam et al., 2000; Simpson et al., 2000). In the studies reporting a protective effect of TNF-α inhibition in paracetamol induced liver injury, the degree of protection was similar to that observed with JNK inhibition in our study (Ishida et al., 2004; Blazka et al., 1995; Blazka et al., 1996). However, the pathogenic role of TNF-α in paracetamol induced hepatic necrosis remains controversial. Studies using blocking antibodies and inhibitors of TNF-α have produced conflicting results, with both protection or no effect reported (Blazka et al., 1995; Blazka et al., 1996; Simpson et al., 2000). Alternatively, some have shown attenuated liver injury in mice lacking TNF receptor expression (TNFR1) while others using a different strain of mice have shown increased liver injury in TNFR1 knockout mice (Ishida et al., 2004; Gardner et al., 2003). Our data suggest that JNK inhibition may limit liver injury via reduced TNF-α expression. In a recent study Kaplowitz and colleagues reported that JNK inhibition limited paracetamol induced liver injury, possibly by interfering with translocation of members of the Bcl2 family into the mitochondrial membrane (Gunawan et al., 2006). This study reported that JNK inhibition will protect TNFR1 knockout mice from paracetamol induced hepatic injury. However, TNF-α expression levels were not measured in this study. These data do not invalidate our postulated mechanism as TNF-α can also induce cell death via TNFR2 signaling (Maeda S et al., 2003; Jupp et al., 2001). Further work is required to fully understand the roles of TNF-α induced TNFR1 and TNFR2 signaling via JNK in paracetamol induced liver failure.
The protective effect of JNK inhibition in paracetamol induced liver injury was not translated into protection in other models of liver injury. JNK inhibition was not protective following Fas ligation and CCL\textsubscript{4} injection and this confirms previous reports (Schwabe et al., 2004; Gunawan et al., 2006). Similar to paracetamol hepatotoxicity, TNF-\(\alpha\) has been implicated in CCL\textsubscript{4} mediated liver injury, but again conflicting published data exist. Injection of soluble TNF receptors limits CCL\textsubscript{4} hepatotoxicity (Czaja et al., 1995) however pre-injection of anti-TNF antibodies has no protective effect (Bruccoleri et al., 1997). Furthermore, studies of TNF receptor 1, 2 or double knockout or TNF-\(\alpha\) knockout mice have demonstrated either no effect or protection following CCL\textsubscript{4} mediated liver injury (Simeonova et al., 2001; Morio et al., 2001; Sudo et al., 2005; Yamada and Fausto, 1998). In contrast to our data with JNK inhibition in the context of paracetamol hepatotoxicity, we observed no reduction in hepatic TNF-\(\alpha\) expression following JNK inhibition and CCL\textsubscript{4} injection (data not shown).

JNK induces the expression of TNF-\(\alpha\) in several cell types (Swantek et al., 1997; Kontoyiannis et al., 1999; Zhu et al., 2005; Shen et al., 2005; Ishizuka et al., 1997). Transfection of liver specific macrophages (kupffer cells) with constitutively active adenovirus expressing the upstream JNK-kinase, MKK-7, induces TNF-\(\alpha\) production and JNK inhibition reduces leptin induced TNF-\(\alpha\) production in the same cell type (Shen et al., 2005). It is noteworthy in view of our data that there is a reported functional redundancy of JNK genes in TNF-\(\alpha\) production from macrophages (Zhu et al., 2005). We speculate that pan-inhibition of the JNK signaling pathway downregulates TNF-\(\alpha\) expression by kupffer cells and reduces paracetamol induced liver injury. However, this may be difficult to study in vivo as kupffer cell depleted mice are protected from paracetamol induced hepatic necrosis (Michael et al., 1999). Future studies with conditional cell specific JNK knockouts in the liver may therefore aid further analysis in this regard.

The liver expresses two JNK genes; JNK 1 and JNK 2. Although complete JNK inhibition by SP600125 and D-JNKI1 was protective, liver injury was similar in JNK\textsubscript{1/-}.
and JNK2−/− mice compared with wild type. Others have recently reported partial limitation of paracetamol induced liver injury in JNK2−/− mice or mice treated with JNK2 antisense RNA, but this protection was not as effective as pan-JNK inhibition with either pharmacological inhibitors or JNK antisense RNA (Gunawan et al., 2006). Context and cell specific effects of JNK1 or JNK2 knockout on cell death have been reported. Our data suggest that redundancy exists between the JNK1 and JNK2 signaling pathways in the context of paracetamol-induced liver injury. We were unable to directly address this further in our study as JNK1−/− JNK2−/− (double null) mice are embryonic lethal, and therefore future studies with conditional knockouts in the liver may aid further analysis of the relative roles of these genes in paracetamol induced hepatic necrosis.

In summary, we found a massive increase in hepatic JNK activity during paracetamol induced hepatic failure in a murine model. Inhibition of hepatic JNK with either a pharmacological or peptide inhibitor significantly reduced liver injury and mortality without affecting paracetamol bio-activation, and confirms and expands recent published data (Gunawan et al., 2006). The hepatoprotective effect of JNK inhibition may be due to a specific reduction in hepatic TNF-α expression. From a clinical standpoint, we have shown JNK inhibition is more efficacious in reducing liver injury at later time points when the traditional antidote NAC is no longer effective. These data demonstrate that JNK plays a crucial role in hepatocyte death following paracetamol poisoning, and suggest that JNK inhibition may find clinical application in the group of patients that present late after overdose or in which timing of the overdose is unclear.
CHAPTER 3

Galectin-3 regulates myofibroblast activation and hepatic fibrosis.

3.1 Abstract

Central to fibrogenesis and the scarring of organs is the activation of fibroblasts into matrix-secreting myofibroblasts. We demonstrate that Galectin-3 expression is up-regulated in established human fibrotic liver disease and is temporally and spatially related to the induction and resolution of experimental hepatic fibrosis. Disruption of the Galectin-3 gene blocks myofibroblast activation and procollagen (I) expression in vitro and in vivo, markedly attenuating liver fibrosis. Addition of exogenous recombinant Galectin-3 in vitro reversed this abnormality. The reduction in hepatic fibrosis observed in the Galectin-3-/- mouse occurred despite equivalent liver injury and inflammation, and similar tissue expression of TGF-β. TGF-β failed to transactivate Galectin-3-/- hepatic stellate cells, in contrast with WT hepatic stellate cells; however, TGF-β-stimulated Smad-2 and -3 activation was equivalent. These data suggest that Galectin-3 is required for TGF-β mediated myofibroblast activation and matrix production. Finally, in vivo siRNA knockdown of Galectin-3 inhibited myofibroblast activation after hepatic injury and may therefore provide an alternative therapeutic approach to the prevention and treatment of liver fibrosis.
3.2 Introduction

Galectins are members of a newly defined and growing family of animal lectins (Barondes et al., 1994a; Barondes et al., 1994b; Kasai and Hirabayashi, 1996; Rabinovich et al., 2002). Galectin-3 is a β-galactoside binding animal lectin of approximately 30 kDa. This unique Galectin is composed of two domains: a carboxyl-terminal domain that contains the carbohydrate-binding region and an amino-terminal domain consisting primarily of tandem repeats of nine amino acids (Liu, 1990) to cross-link carbohydrate and non-carbohydrate ligands. Galectin-3 is a pleiotropic molecule found in the nucleus, cytoplasm and at the cell surface and can also be secreted by an unorthodox mechanism that bypasses the endoplasmic reticulum and the golgi (Mehul and Hughes, 1997). *In vitro* Galectin-3 has been implicated in a variety of biological processes including cell growth and proliferation (Moutsatsos et al., 1987; Inohara et al., 1998), adhesion (Kuwabara and Liu, 1996; Inohara and Raz, 1995; Inohara et al., 1996) and cell survival (Yang et al., 1996; Akahani et al., 1997). Increased Galectin-3 expression has been noted in tissue fibrosis (Kasper and Hughes, 1996; Hsu et al., 1999; Wang et al., 2000) and *in vitro* exogenous Galectin-3 stimulates myofibroblast proliferation (Sasaki et al., 1999; Maeda N, 2003). However the relevance of these observations to the pathogenesis of tissue fibrosis *in vivo* has not been examined.

Initial *in vivo* studies demonstrated that Galectin-3 knockout mice have attenuated peritoneal inflammatory responses to thioglycollate instillation (Colnot et al., 1998; Hsu et al., 2000), which suggests a role for Galectin-3 in the development of acute inflammation. However the mechanistic role of Galectin-3 in tissue fibrosis has not been addressed. The mechanisms that are involved in fibrogenesis are distinct from those involved in inflammation. Although inflammation typically precedes fibrosis, experimental models now show that the amount of fibrosis is not necessarily linked with the severity of inflammation (Strieter et al., 2004). To understand further the mechanisms underlying liver fibrosis we examined the hypothesis that Galectin-3 controls myofibroblast activation and collagen deposition in an experimental model of hepatic fibrosis using mutant mice lacking the gene encoding Galectin-3.
3.3 Materials and Methods

Materials
Tissue culture reagents were purchased from Life Technologies (Paisley, Scotland, UK). Tissue culture plastics were obtained from Costar (Loughborough, Leicestershire, UK) and Falcon (Runcorn, Cheshire, UK). Cytokines and recombinant mouse Galectin-3 were purchased from R&D Systems (Abingdon, Oxon, UK) and Peprotech EC Ltd (London, UK). All other reagents were from Sigma-Aldrich Company Ltd (Poole, Dorset, UK) unless otherwise stated.

Animals
Mice were maintained in 12-hour light/12-hour dark cycles with free access to food and water. All procedures were performed in accordance with Home Office guidelines [Animals (Scientific Procedures) Act 1986]. Generation of Galectin-3−/− mice by gene-targeting technology has been described previously (Colnot et al., 1998). As controls, age and sex-matched wild type littermate mice were used.

Human biopsy specimens
Archival human liver samples were obtained from the University of Edinburgh Department of Pathology files. Samples had been fixed in 4% paraformaldehyde and embedded in paraffin wax.

Reversible model of CCL4 induced liver fibrosis
A reversible model of CCL4 induced liver fibrosis was undertaken as previously described (Issa et al., 2004). Liver fibrosis was induced by the twice weekly injection of CCL4 in olive oil vehicle in male Sprague Dawley rats (250–500 g) for 12 weeks, after which animals were killed and their livers harvested for analysis at peak fibrosis (3 days after the final dose of CCL4) and at 168 days of spontaneous recovery (n = 4–6 for each cohort at each time point). After harvesting, livers were divided with a minimum of 2 lobes
fixed in formalin for histologic analysis and immunohistochemistry and the remaining liver snap-frozen for protein and mRNA analysis.

**Acute CCL₄ Model**

Acute self-limiting liver injury characterized by stellate cell activation was induced by I.P. injection of CCL₄. After overnight fast (with free access to water) mice were injected I.P. with 1 ul/g body weight sterile CCL₄ in a 1:3 ratio with olive oil. After a single dose, animals were anaesthetised, blood taken and their livers harvested for analysis at 24, 48, 72, 96 and 168 hours (n = 6 in each group at each time point). Cohorts of six animals per time-point were also treated with olive oil vehicle as controls.

**Chronic CCL₄-induced liver fibrosis model**

Mice (n=6 in each group) were injected I.P. with 1 ul/g body weight sterile CCL₄ in a 1:3 ratio with olive oil twice weekly for eight weeks. A control group was treated in parallel with olive oil vehicle. Tissues were harvested twenty four hours after the last injection of CCL₄.

**Immunohistochemistry**

Paraffin-embedded sections of human and mouse tissue were processed for immunohistochemistry as described (Iredale *et al.*, 1998) using the following primary antibodies: mouse monoclonal anti-αSMA clone 1A4 (Sigma, Poole, UK), rat monoclonal anti-mouse Galectin-3 clone 8942F (Cedarlane, Ontario, Canada), mouse monoclonal anti-human Galectin-3 clone 9C4 (Novostra, Newcastle Upon Tyne, UK), rabbit anti-rat Galectin-3 (a kind gift from FT Liu, University of California, Davis, USA), rat anti-mouse Gr-1 mAb clone RB6 8C5 (BD PharMingen, UK), rat anti-mouse F4/80 clone C1:A3-1 (Serotec, UK). Species appropriate isotype control antibodies were also used for each experiment.
Immunofluorescence
Sections were dewaxed, taken through graded alcohols to PBS and microwave antigen retrieved in sodium citrate. A biotin-blocking step was used (DAKO X0590). Slides were pre-incubated in normal rabbit serum (DAKO) at 1/25 dilution in PBS for 10 minutes. The slides were then incubated in mouse monoclonal α-SMA (Clone 1A4, A-2547, Sigma, Poole, UK) at a dilution of 1/4000 in PBS (35 minutes) at room temperature. The secondary antibody (biotinylated rabbit anti-mouse, DAKO, E0354) was applied for 35 minutes at room temperature. As a tertiary layer streptavidin-alkaline phosphatase (AP) (DAKO D0396) was diluted to 1/50 in PBS, for 35 minutes at room temperature. Sections were washed in PBS between each ab layer, and Vector Red substrate (Vector Laboratories SK 5100, Peterborough, UK) was applied for 15 minutes at room. Next the slides were incubated in normal goat serum (DAKO) at 1/25 dilution in PBS for 10 minutes then rat anti-MAC-2 was used at 1:200 dilution for 35 minutes. Slides were washed in PBS then incubated in goat anti-rat FITC conjugated 1:100 antibody for 35 minutes. Slides were again washed in PBS prior to mounting in Vestashield (Vector Laboratories).

Determination of fibrosis
Tissue fibrosis was visualized and quantified with a picrosirius red stain as described previously (Issa et al., 2004). Digital image analysis (as described below) was used to quantitate the amount of red-stained collagen fibers.

Digital image analysis
Morphometric measurements were made on 10 µm sections stained with picrosirius red using OpenLab software (Improvision, Coventry, UK). Forty random fields (captured with a Leica DMLB microscope, Leica DC300 camera and Leica image manager) from each section were analysed at a final magnification of x100. Each captured field was analyzed by separation into red, green and blue (RGB) filters and the red area was mathematically divided by the RGB area and multiplied by 100%. This represents the percentage area staining positively for collagen fibers, providing a quantitative value on a continuous scale.
**Hepatic stellate cell extraction and culture**

Primary human HSC (extracted from the margins of normal human liver resected for colonic metastatic disease) and mouse HSC were extracted from human and mouse liver by pronase and collagenase digestion and purified by density gradient centrifugation. HSCs were then culture-activated in the presence of serum on uncoated tissue-culture plastic and passaged exactly as previously described (Issa *et al*., 2004; Iredale *et al*., 1998). In more detail, the protocol for primary mouse HSC isolation is listed below:

Enzymes were made up as follows - Collagenase 30mg into 15 mls HBSS(+) and sterile filter, Pronase 180mg into 15 mls HBSS(+) and sterile filter (may take some time to dissolve) and DNase 40mg into 40 mls HBSS(+) and sterile filter. Then take 5 mls of collagenase solution and 5 mls pronase solution, mix and to 5 mls of this add 500 ul of heparin (5,000iu/ml). Make up Complete DMEM (16 % FCS, Pen/strep/gentamycin).

Then prepare X2 150 ml sterilins (if doing two groups of mice) with 50-60 mls HBSS(+) in each to transport livers, 5 mls of enzyme/heparin mix and 1 ml syringes and 30 G needles. Open peritoneal cavity, cannulate portal vein with 30 G needle, then cut IVC and then flush liver with enzyme/heparin mix (liver should blanch). Once livers isolated, add together residual 10 + 10 mls of pronase and collagenase and make upto 40-50 mls in HBSS(+). Chop up livers with ethanol sterilised scissors. Wrap falcons in parafilm and put in shaker @37\(^{\circ}\)C, 220 rpm for 20 mins. Make DNase upto 40 mls with HBSS(+). Add 5 mls of DNase to liver digest and pasteur pipette up and down. Pour digest through nybolt filter and distribute round filter with sterile 10ml stripette. Wash through with HBSS(+) but keep total amount collected in beaker <100mls. Split into 2 falcons (50 mls each) and spin for 7 mins. @ 1,800 rpm. Resuspend pellets in 2-3 mls DNase and make upto 50 mls again with HBSS(+) and spin again for 7 mins. @ 1,800 rpm. Resuspend pellet in 2-3 mls DNase then pool samples into same Falcon and make upto 8.9 mls total volume with HBSS(+).

Prepare Optiprep: Make sure centrifuge has been set to chill (4\(^{\circ}\)C) and BRAKE is OFF. Add 5.9 mls of neat optiprep to the 8.9 mls of cell suspension and mix. Into a 15 ml Falcon put 4.8 mls neat optiprep plus 3.2 mls HBSS(+) and mix. Then divide this into two 15 ml Falcons (4 mls each) – this is bottom layer of gradient. Now take 7.4 mls of
cell suspension mix and gently layer onto bottom layer of both 15 ml Falcons. Now layer onto both tubes 0.5 mls HBSS(+). Spin this at 2,500 rpm (1,400g) for 20 mins in chilled centrifuge with NO BRAKE. Take top HSC layer and add 1.5 mls DNAse and make up to 50 mls with HBSS(+). Spin @ 400g (1,800 rpm) for 7 minutes at room temp. Resuspend pellet in 10 mls complete DMEM. Cell count 1:1 ratio e.g. 50 ul cell suspension/50 ul trypan blue. Count cells present in 16 squares on haemocytometer. No. of cells in 16 squares x Y x 10,000 = no. of cells/ml, x no. of mls (10 in this case). Where Y is dilution factor (in this case 2). If for example 20 million HSCs harvested, split into 4 flasks with 12 mls complete media in each flask.

Extracted HSC were cultured on plastic until they were activated to a myofibroblastic phenotype after 3-7 days. Human and mouse HSC were used for experiments after activation in primary culture or before fourth passage. Cells were cultured in Dulbecco's modified Eagle's medium in the presence of 16% fetal calf serum and antibiotics.

**BMDM preparation and cytokine measurement**

BMDMs were prepared from WT and *Galectin-3*⁻/⁻ mice as described previously (Duffield et al., 2000). Mature BMDMs (5x10⁵/well) were added to the wells of 24-well plates. After 3h the wells were washed to remove non-adherent cells. Wells were treated with lipopolysaccharide (LPS) (100ng/ml) and murine interferon-γ (IFNγ) (100 U/ml) in serum free media. After 24 hrs incubation the supernatants were harvested and clarified by centrifugation at 10000g for 5 minutes and frozen at -80°C. Cytokine secretion was determined by a cytometric bead array, performed according to the manufacturers instructions (BD Pharmingen). TGF-β and TNF-α levels were measured by ELISA as per manufacturers instructions (Quantikine, R & D systems, Abington, UK).

**Western Blotting**

Western blot analysis of mouse liver tissue and human and mouse HSC lysates was undertaken using the following primary antibodies; mouse monoclonal anti-α-SMA antibody clone 1A4 (Sigma, UK), mouse monoclonal anti-Galectin-3 antibody clone A3A12 (Alexis Biochemicals, UK), rabbit polyclonal anti-Smad 2 and 3 antibody
(Biosource, UK), rabbit polyclonal anti-β-actin antibody (Sigma, UK), anti-phospho ERK1/2 (Sigma, UK), anti-phospho PKC (Cell signaling technology, USA).

**HSC growth**

Primary mouse HSC were seeded at a density of 5,000 cells per well in 96 well plates and proliferation at various time points was measured using MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl tetrazolium bromide) formazan production (Sigma, UK) as per manufacturer’s instructions.

**Alanine aminotransferase determination**

Blood was centrifuged at 4°C and the serum stored at -80°C until use for determination of alanine aminotransferase levels by an automated enzyme assay (Olympus 20700 analyser).

**Preparation of siRNAs**

siRNAs were synthesised using 5'-silyl-2'-ACE method (Dharmacon, Colorado, USA). The sense and anti-sense strands of mouse siRNA were: *Galectin-3* (sequence 1), beginning at nt 502, 5’- GAUGUUGCCUUCACUUUAUdGdTdT-3’ (sense), 5’-P.UUAAGUGGAAGCGAACCAUCdTdT-3’ (antisense); *Galectin-3* (sequence 2), beginning at nt 4, 5’- GCAGACACGCUUUUCGCUUAdTdT-3’ (sense), 5’-P.UAAAGCGAAAGCGUUGCUGCdTdT-3’ (antisense); *Galectin-3* (sequence 3), beginning at nt 678, 5’- GGUCAACAGCUUUACGCUUAdTdT-3’ (sense), 5’-P.UAAGGCGGAAGCGUUGCUGCdTdT-3’ (antisense); *Galectin-3* (sequence 4), beginning at nt 190, 5’- GGACAGGCUCUCCUAGUGdTdT-3’ (sense), 5’-P.CACUAGGAGGACGCGUUGCCdTdT-3’ (antisense); P represents phosphate. The sense and anti-sense strands of human siRNA were: *Galectin-3* (sequence 1), beginning at nt 550, 5’ – GAAGAAAGACAGUCGGUUDdTdT-3’ (sense), 5’-P.AAAGACACGCUUUACGCUUdTdT-3’ (antisense); *Galectin-3* (sequence 2), beginning at nt 518, 5’- GCAUAACAAACCGUGGAUAAdTdT-3’ (sense), 5’-P.UUAUCAACACCGUUGAUGCdTdT-3’ (antisense); *Galectin-3* (sequence 3), beginning at nt 660, 5’- GUACAAUCAUCGGUUAAAdTdT-3’ (sense), 5’-P.
UUUAACCGAUUGUACdTdT-3’ (antisense); *Galectin-3* (sequence 4), beginning at nt 658, 5’- CAGUACAAUCAUCGGGUUAdTdT (sense), 5’-P. UAACCCGAUGAUUGUACUGdTdT-3’ (antisense). Control duplex was siCONTROL non-targeting siRNA #2 (Dharmacon, Colorado, USA).

**siRNA treatment in vitro**
Subconfluent cultures of primary mouse and human HSC in 6 well plates were transfected with siRNA (final concentration 250 nM) using oligofectamine as per manufacturers instructions (Invitrogen, UK). After 96 hours cells were lysed for protein or RNA extraction.

**siRNA treatment in vivo**
Acute CCL4 hepatic injury model: Male 129/Sv mice aged 8–10 weeks and weighing 20–25 g were given CCL4 in a 1:3 ratio with olive oil I.P. Two hours later animals were anaesthetized and a cannula inserted into the portal vein. 50 µg of Galectin-3 siRNA (Duplex # 2, siSTABLE, Dharmacon, Colorado, USA) in 300 ul PBS was rapidly delivered via the portal vein (approximately 2mg/kg). Prior to removal of the cannula, Gelaspon (Johnson & Johnson, UK) was applied to the portal vein to prevent bleeding. Further delivery of Galectin-3 siRNA at 24 and 48 hours was by rapid hydrodynamic tail vein injection (50 ug of siRNA in 1ml PBS). Control mice received PBS or siCONTROL non-targeting siRNA #2 (Dharmacon, Colorado, USA) via the same routes. Mice were anaesthetised on day 3 (*n* = 6 in each group), and liver tissue fixed in buffered formalin and the remaining liver snap-frozen for protein and mRNA analysis.

**Real-time PCR**
Total RNA from whole liver was reverse transcribed into cDNA using random hexamers and the Taqman multiscrIBE RT kit as per manufacturers instructions (Applied Biosystems, Warrington, UK). Mouse primers and probes were as follows: Procollagen(I), forward 5’-TTCACTACAGCAGCGGGTG-3’, reverse 5’-GATGACTGTCTTGCCCCAAGTT-3’ and probe FAM 5’-ATGGGCTGCAGGAGTCACA -3’ TAMRA. For mouse α-SMA forward primer
Statistical analysis

Results are presented as means ± S.E.M. Significance of the differences between means was assessed using one-way analysis of variance (ANOVA) or two-tailed Student's $t$ test. Values of $P < 0.05$ were considered significant. Unless stated otherwise, studies were performed on three to six independent occasions.
3.4 Results

Galectin-3 expression is upregulated in human liver fibrosis and is temporally and spatially related to fibrosis in a reversible rat model of liver fibrosis.

Galectin-3 expression was examined by immunohistochemistry in normal and fibrotic human liver (Fig.1a, b). In established human liver fibrosis, regardless of aetiology (hepatitis B or C, autoimmune, copper or iron overload, primary biliary cirrhosis or alcohol-induced), Galectin-3 expression was dramatically increased in the cirrhotic nodules of hepatocytes, particularly at the periphery of the nodules (n=36 cases). Furthermore, Galectin-3 expression was examined in a well-established rat model of reversible carbon tetrachloride (CCL4) induced liver fibrosis (Issa et al., 2004; Iredale et al., 1998) (Fig.1c). Galectin-3 expression was temporally and spatially associated with fibrosis (collagen fibers stained red with picrosirius red), minimal in normal rat liver, maximal at peak fibrosis and was virtually absent again at 24 weeks (recovery from fibrosis). These data suggest that the development (and resolution) of fibrosis in different organs may be regulated by Galectin-3.
Figure 1

Normal

Fibrosis

Collagen

Gal-3

Control 12 weeks CCL$_4$ 24 weeks post CCL$_4$
Figure 1
Galectin-3 expression is upregulated in human liver fibrosis. (a) Galectin-3 expression in bile duct epithelium and kupffer cells (tissue macrophages) in normal human liver. (b) Galectin-3 in cirrhosis secondary to hepatitis C infection. Dense staining is present in the cirrhotic nodules. Scale bar 400 µm. Galectin-3 expression is temporally and spatially related to fibrosis in a reversible rat model of liver fibrosis. (c) Upper panels: Collagen stained with picrosirius red. Scale bar, 100 µm. Lower panels: Galectin-3 immunohistochemistry. Scale bar, 200 µm. Control (olive oil vehicle only, left panels). Peak fibrosis: Rat liver following 12 weeks of twice-weekly I.P. CCL₄ (middle panels). Resolution: 24 weeks after cessation of CCL₄ induced liver injury (right panels).
Galectin-3 plays a critical role in liver fibrosis.

The significance of the induction of Galectin-3 expression in the development of liver fibrosis was examined using CCL4 induced liver injury as a model system for liver fibrogenesis. To assess the expression pattern of Galectin-3 in experimental liver fibrosis we examined liver tissue from wild type (WT) mice which had received either olive oil (control) or CCL4 intraperitoneally twice weekly for eight weeks. Following eight weeks of CCL4 treatment, increased Galectin-3 expression was observed in the periportal areas and areas of bridging fibrosis in the liver. Particularly dense Galectin-3 staining was noted at the periphery of the inflamed damaged areas. Galectin-3 was only expressed in bile duct epithelia and kuffer cells in the liver from control (olive oil treated) animals (Fig.2a). Real-time PCR for Galectin-3 mRNA showed a marked increase in expression with the development of hepatic fibrosis in the CCL4 treated animals compared with control (Fig.2b left panel). This increase in mRNA expression was paralleled by an increase in hepatic Galectin-3 protein expression (Fig.2b right panel). The absence of Galectin-3 expression in Galectin-3−/− mice was confirmed both by immunohistochemistry and western blotting following eight weeks of CCL4 treatment (results not shown).

Hepatic collagen was stained with picrosirius red (Fig.2c) and quantified using digital image analysis (Fig.2d left panel). Striking collagen deposition in the same distribution as Galectin-3 was present in the periportal areas and areas of bridging fibrosis in the WT animals. By contrast there was significantly less collagen deposition in the Galectin-3−/− mice (P<0.05). Transcripts for procollagen(I) mRNA (collagen(I) is highly expressed in human and animal models of liver fibrosis) were assessed using real-time PCR (Fig.2d right panel) and were significantly decreased in the livers from Galectin-3−/− compared with WT mice following chronic CCL4 treatment (P<0.05). This suggests that Galectin-3 regulates hepatic collagen deposition during repeated CCL4 induced liver injury. We therefore went on to examine the mechanism underlying this important observation.

The hepatic stellate cell (HSC) is the key fibrogenic cell of the liver and represents a paradigm cell type in studies of the pathogenesis of tissue fibrosis (Gressner, 1996;
Following any cause of liver injury, quiescent HSC undergo activation to proliferative, fibrogenic and contractile myofibroblasts with increased expression of $\alpha$-smooth muscle actin ($\alpha$-SMA), a widely accepted marker of myofibroblast activation (Friedman, 2000; Bataller and Brenner, 2005) \textit{in vitro} and \textit{in vivo}. Therefore, to investigate the role of Galectin-3 in liver fibrogenesis, we assessed $\alpha$-SMA expression as a measure of HSC activation in WT and Galectin-3$^{-/-}$ mice after eight weeks of CCL$_4$ or olive oil (control). $\alpha$-SMA expression was markedly increased in WT compared with Galectin-3$^{-/-}$ mice with the same temporal and spatial distribution as Galectin-3 and collagen expression following eight weeks of CCL$_4$ administration (Fig.2e). There was significantly less $\alpha$-SMA expression in the Galectin-3$^{-/-}$ mice compared with WT quantified using digital image analysis (Fig.2f left panel, $P<0.01$). The transcripts for $\alpha$-SMA mRNA, as assessed by real-time PCR, were significantly increased in WT animals following CCL$_4$ treatment compared with animals which received olive oil (control) (Fig.2f right panel), indicating HSC activation in this model of chronic liver injury. However $\alpha$-SMA transcripts were significantly decreased in Galectin-3$^{-/-}$ mice compared with WT ($P<0.01$). This decrease in mRNA expression was paralleled by a decrease in hepatic $\alpha$-SMA protein expression as assessed by western blot analysis of whole liver tissue (Fig.2f right inset). These data indicate that Galectin-3 mediated activation of HSC \textit{in vivo} is a central mechanism underlying hepatic fibrosis. Furthermore, we investigated myofibroblast activation in models of renal fibrosis and pulmonary fibrosis using unilateral ureteric obstruction and intratracheal instillation of silica respectively (unpublished observations). In both instances we found defective myofibroblast activation in the Galectin-3$^{-/-}$ mice. This demonstrates the broad applicability of our results and potential relevance to many forms of tissue fibrosis in different disease states.
Figure 2

a) WT

Galectin-3

Control

CCL$_4$

b) Galectin-3/18S

Gal-3

β-actin

Control

CCL$_4$

c) WT

Galectin-3$^{-/-}$

Collagen
Figure 2

d

% Collagen

WT  Galectin-3^{-/-}  CCL_4  CCL_4

Procollagen(I)/18S

WT  Galectin-3^{-/-}  CCL_4  CCL_4

e

WT  Galectin-3^{-/-}

α-SMA
Figure 2
Galectin-3 plays a critical role in organ fibrosis.
Mice were treated with olive oil (control) or CCL₄ I.P. twice weekly for eight weeks. (a) Galectin-3 expression in control (left) and following chronic CCL₄ treatment (right) in WT mouse liver (n=6 mice in each group). Scale bar, 400 µm. (b) Left panel: Real-time PCR quantitation of Galectin-3 expression in whole liver homogenates from control (olive oil vehicle) and chronic CCL₄ treated mice. *** (P < 0.0001 compared with control). Right panel: Representative Galectin-3 and β-actin western blots of whole liver from control and CCL₄ treated wild type mice (n=6 mice in each group). (c) Collagen staining with picrosirius red of liver tissue following chronic CCL₄ treatment of WT and Galectin-3⁻/⁻ mice (n=6 mice in each group). Scale bar, 200 µm. (d) Upper panel: Quantitation of collagen staining using digital image analysis. *P <0.05. Lower panel: Real-time PCR quantitation of procollagen(I) in whole liver homogenates from chronic CCL₄ and control groups. * (P <0.05 compared with WT). (n=6 mice in each group). (e) α-SMA staining of liver tissue following chronic CCL₄ treatment (n=6 mice in each group). Scale bar, 200 µm. (f) Upper panel: Quantitation of α-SMA staining using digital image analysis. ** (P <0.01 compared with WT). Lower panel: Real-time PCR quantitation of α-SMA in whole liver homogenates in chronic CCL₄ and control groups. * (P <0.05 compared with WT) (n=6 mice in each group). Inset: Representative western blots of α-SMA and β-actin expression in whole liver homogenates from chronic CCL₄ treated mice.
Disruption of the Galectin-3 gene does not affect initial liver injury or inflammatory cell infiltrate following CCL4 treatment.

Following a single I.P. injection of CCL4 initial hepatic injury as judged by alanine aminotransferase (ALT, a marker of hepatocyte damage) was similar in WT and Galectin-3−/− mice at days 1-7 (Fig. 3a,b). Furthermore total liver tissue levels of the pro-inflammatory cytokine TNF-α in WT and Galectin-3−/− mice measured by ELISA were not significantly different 24 hours post CCL4 liver injury (WT 1.4 ± 0.22 ng TNF-α/µg whole liver protein, Galectin-3−/− 1.65 ± 0.3 ng TNF-α/µg whole liver protein (n=6 mice in each group, p > 0.05, data not shown). Quantitation of hepatic inflammatory cell recruitment (as assessed by cell counts of macrophages and neutrophils) was similar in WT and Galectin-3−/− mice at days 1-7 (Fig.3c,d). Furthermore, there was no difference in T lymphocyte recruitment between WT and Galectin-3−/− mice as assessed by CD3 immunohistochemistry and counting. The macrophage in particular is an important inflammatory cell involved in the pathogenesis of tissue fibrosis (Duffield et al., 2005; Imamura et al., 2005) and therefore we examined pro-inflammatory cytokine release from WT and Galectin-3−/− bone marrow derived macrophages. Maturation of WT and Galectin-3−/− bone marrow derived macrophages demonstrated equivalent expression of CD11b and F4/80 (Fig. 3e). Following activation with IFN-γ/LPS no significant difference was observed in the release of the pro-inflammatory cytokines TNF-α or IL-6 between WT and Galectin-3−/− macrophages (Fig.3f). These data demonstrate that the difference in liver fibrosis observed between the two genotypes is not secondary to a difference in initial tissue injury, inflammatory cell recruitment or macrophage pro-inflammatory cytokine release.
Figure 3

(a) WT vs. Galectin-3 \(-/-\)

Control

Acute CCL\(_4\)

(b) ALT (IU/L)

WT CCL\(_4\) vs. Galectin-3 \(-/-\) CCL\(_4\)
Figure 3

c

Macrophage count (cells/HPF)

Day

WT

G-3/-

d

Neutrophil count (cells/HPF)

Day

WT

G-3/-
Figure 3

e

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88
Figure 3

**TNF-α**

![Graph showing TNF-α levels.

**IL-6**

![Graph showing IL-6 levels.](image)
Figure 3

Disruption of the Galectin-3 gene does not affect initial liver injury or inflammatory cell infiltrate following CCL4 treatment.

Mice were treated with a single I.P. injection of CCL4 or olive oil control and livers harvested at days 1, 2, 4 and 7 (n=6 mice in each group). (a) Haemotoxylin and eosin staining of livers 24 hrs after vehicle (olive oil) or CCL4 treatment in WT and Galectin-3<sup>-/-</sup> mice. (b) Quantitation of serum alanine aminotransferase (ALT) release 24 hrs after acute CCL4 induced liver injury. (c) Macrophage infiltration in WT (filled bars) and Galectin-3<sup>-/-</sup> (open bars) livers in control (day 0) and 1, 2, 4 and 7 days post CCL4 induced liver injury measured by F4/80 staining and cell counting. (d) Neutrophil infiltration in WT (filled bars) and Galectin-3<sup>-/-</sup> (open bars) livers in control (day 0) and 1, 2, 4 and 7 days post CCL4 induced liver injury measured by Gr-1 staining and cell counting. (e) FACS characterisation of WT and Galectin-3<sup>-/-</sup> BMDMs matured for 7 days using F4/80 and CD11b. (f) Pro-inflammatory cytokine profiles of WT and Galectin-3<sup>-/-</sup> BMDMs activated with IFN-γ/LPS.
Myofibroblast activation is Galectin-3 dependent.

HSC activation to a myofibroblast phenotype is a critical event in extracellular matrix deposition and cirrhosis (Friedman, 2000; Bataller and Brenner, 2005). We observed dual staining of Galectin-3 and α-SMA within cells in areas of fibrotic liver injury following eight weeks of CCl₄ treatment in WT mice. This demonstrates that Galectin-3 expression is up-regulated in myofibroblasts during the injury response in vivo (Fig.4a). In vitro Galectin-3 expression was upregulated on transition from the quiescent to the myofibroblastic phenotype in both primary murine HSC and primary human HSC when activated on tissue culture plastic (Fig.4b). This well-established and validated in vitro method of HSC activation closely models in vivo myofibroblast activation (Issa et al., 2004; Iredale et al., 1998) and has been used extensively to model and examine the changes which take place during the phenotype switch of fibroblasts to extracellular matrix secreting contractile myofibroblasts. To assess whether myofibroblast activation is dependent on Galectin-3, HSC from WT and Galectin-3⁻/⁻ mice were cultured for 7 days and their activation status assessed by western blot analysis for α-SMA expression. At day 7, protein expression of α-SMA was significantly decreased in Galectin-3⁻/⁻ HSC compared with WT HSC (Fig.4c). Addition of exogenous recombinant murine Galectin-3 to Galectin-3⁻/⁻ HSC in vitro reversed the Galectin-3⁻/⁻ phenotype resulting in increased α-SMA expression assessed by western blotting (Fig.4d). Rescue of the pro-fibrotic phenotype by addition of exogenous Galectin-3 was further confirmed with real-time PCR which demonstrated upregulation of procollagen(I) expression (Fig.4e, P<0.05). WT primary murine HSCs were shown to proliferate faster than Galectin-3⁻/⁻ HSC by an MTT assay when plated on tissue culture plastic ex-vivo. This defect in proliferation of the Galectin-3⁻/⁻ HSC could be restored by the addition of recombinant murine Galectin-3 (Fig.4f). A time-course experiment was undertaken to determine whether exogenous Galectin-3 is internalised or membrane-bound when added to primary murine HSC. Figure 4g shows that Galectin-3 is rapidly internalised (within 10 minutes) when added to Galectin-3⁻/⁻ HSC and very little Galectin-3 accumulates at the membrane.
Figure 4

a

Control

i

Galectin-3

ii

\( \alpha \)-SMA

iii

Merged image

CCL\(_4\)

iv

Galectin-3

v

\( \alpha \)-SMA

vi

Merged image

b

mGal-3

\[
\begin{array}{c}
\text{Day} \\
0 \quad 2 \quad 7 \quad 10
\end{array}
\]

hGal-3

\[
\begin{array}{c}
\text{Day} \\
0 \quad 7
\end{array}
\]

c

\[
\begin{array}{c}
\text{WT} \quad \text{Gal-3\textsuperscript{-/-}}
\end{array}
\]

\[
\begin{array}{c}
\text{16\% FCS}
\end{array}
\]

\( \alpha \)-SMA

\( \beta \)-actin
Figure 4

d
\[ \alpha -\text{SMA} \rightarrow \text{WT} +rG-3 \rightarrow \text{Gal-3}^{-/-} \]
\[ \beta -\text{actin} \rightarrow 16\% \text{ FCS} \]

\[ 16\% \text{ FCS} \]

\[ \text{Procollagen(I)/18S} \]

e

\[ \text{WT} +rG-3 \rightarrow \text{Gal-3}^{-/-} \]

\[ 16\% \text{ FCS} \]

\[ * \]

f

\[ \% \text{ Growth} \]

\[ 0 \quad 2 \quad 4 \quad 6 \quad 8 \quad \text{Time (days)} \]
Figure 4

g

i

ii

iii

iv
Figure 4  
Myofibroblast activation is Galectin-3 dependent.  
(a) Galectin-3 expression is up-regulated in myofibroblasts during the hepatic fibrotic response in vivo. Liver sections from WT control (olive oil) (i-iii) and chronic CCL4 injured (8 weeks) (iv-vi) mice were stained with rat anti-mouse Galectin-3 antibody (green), mouse monoclonal anti-αSMA (red) and DAPI (blue). (b) Western blot analysis of Galectin-3 expression in primary mouse (mGal-3) and primary human (hGal-3) HSC during transition from the quiescent to the activated phenotype on tissue culture plastic. (c) Western blot analysis of α-SMA and β-actin expression in WT and Galectin-3−/− primary mouse HSC cultured on tissue culture plastic for 7 days in 16% FCS. (d) Western blot analysis of α-SMA in WT and Galectin-3−/− primary mouse HSC following addition of recombinant murine Galectin-3 (30ug/ml) to Galectin-3−/− HSC in 16% FCS. (e) Real-time PCR quantitation of procollagen(I) in WT and Galectin-3−/− primary mouse HSC following addition of recombinant murine Galectin-3 (30ug/ml) to Galectin-3−/− HSC in 16% FCS. * (P <0.05 compared with untreated Galectin-3−/− HSC). (f) Cell growth of WT (○), Galectin-3−/− (□), and Galectin-3−/− HSC plus recombinant murine Galectin-3 (30ug/ml) (■) measured by MTT assay. Results represent the mean ± SEM of n=3 experiments. (g) Recombinant murine Galectin-3 internalisation by Galectin-3−/− primary mouse HSC. Cells were stained with DAPI (blue) and rat anti-mouse Galectin-3 antibody (green). (i) Untreated, (ii) 10 min, (iii) 30 min, (iv) 60 min following addition of 30 ug/ml recombinant mouse Galectin-3.
To further study the role of Galectin-3 in myofibroblast activation and tissue fibrosis, a rationally designed siRNA pool targeting Galectin-3 mRNA was used to inhibit Galectin-3 expression in WT primary murine HSC. HSC were isolated, activated on tissue culture plastic and treated with phosphate buffered saline (PBS), liposome formulated non-targeted control duplex or liposome formulated Galectin-3 siRNA. Galectin-3 siRNA treatment reduced Galectin-3 mRNA expression 10-fold as compared with control siRNA or mock treatment as assessed by quantitative real-time PCR (Fig.5a, \( P<0.0001 \)). siRNA knock-down of Galectin-3 in HSC did not affect cell viability as assessed by trypan blue exclusion. This marked inhibition of Galectin-3 expression resulted in a significant reduction in both \( \alpha \)-SMA and procollagen(I) mRNA expression as assessed by real-time PCR (Fig.5b, c respectively). Western blot analysis confirmed almost complete inhibition of Galectin-3 expression after targeted siRNA treatment relative to appropriate control treatments (Fig.5d, upper panel). Western blot analysis also confirmed the reduction in \( \alpha \)-SMA expression at the protein level (Fig.5d, middle panel). An important requirement for attributing functional outcomes to inhibition of the targeted gene in siRNA experimentation is to verify the effects with multiple gene targeting siRNAs. To this end four independent murine Galectin-3 targeting siRNA duplexes (as listed in materials and methods) were shown to give similar results (data not shown). To further assess the potential clinical applicability of Galectin-3 siRNA as a therapy in human fibrotic liver disease, primary human HSC were transfected with human Galectin-3 targeting siRNA duplexes. Western blot analysis again showed a marked inhibition of Galectin-3 and \( \alpha \)-SMA expression (Fig.5e) and real-time PCR demonstrated a reduction in mRNA transcripts for procollagen(I) (Fig.5f). These data further support our \textit{in vivo} findings that defective myofibroblast activation is the mechanism mediating reduced hepatic fibrosis observed in the \textit{Galectin-3} \(-/-\) mouse.
Figure 5

a) Galectin-3/18S

b) α-SMA/18S

c) Procollagen(I)/18S

d) Mouse

   - Gal-3
   - α-SMA
   - β-actin

   CD 125 250 siRNA nM

f) Procollagen(I)/18S

   - PBS CD siRNA

β-actin

***
Figure 5
Galectin-3 siRNA inhibits myofibroblast activation and procollagen(I) expression in HSC.

Real-time PCR quantitation of (a) Galectin-3 expression, (b) α-SMA expression and (c) procollagen(I) expression in PBS, control duplex (CD) or Galectin-3 siRNA (250 nM) treated primary mouse HSC. *** ($P < 0.0001$ compared with control duplex). (d) Western blot analysis of Galectin-3, α-SMA and β-actin expression in primary mouse HSC 96 hours post transfection. (e) Western blot analysis of Galectin-3, α-SMA and β-actin expression in primary human HSC 96 hours after treatment with PBS, control duplex (CD) or Galectin-3 siRNA (siRNA). (f) Real-time PCR quantitation of procollagen(I) expression in primary human HSC 96 hours after treatment with either PBS, control duplex (CD) or Galectin-3 siRNA (siRNA) *** ($P < 0.0001$ compared to control duplex). Bars represent the mean ± SEM of three to six independent experiments.
Galectin-3 regulates myofibroblast activation and hepatic fibrosis despite similar levels of TGF-β expression and signaling.

TGF-β is a major pro-fibrogenic cytokine involved in the pathogenesis of fibrosis in many different organ systems (Leask and Abraham, 2004). Blocking TGF-β \textit{in vivo} using antagonists of the TGF-β type 2 receptor or adenoviral administration of dominant negative versions of the receptor have effectively inhibited the fibrotic response to injury in a number of organs (Uemura \textit{et al.}, 2005; Qi \textit{et al.}, 1999). However, mechanisms of tissue fibrosis also exist that are TGF-β independent both in the liver and other organs (Kaviratne \textit{et al.}, 2004; Rodriguez-Vita \textit{et al.}, 2005). To determine whether the reduced hepatic fibrosis observed in the Galectin-3\textsuperscript{-/-} mice compared with WT may be secondary to alterations in the expression of TGF-β, we measured TGF-β mRNA expression in whole liver samples using real-time quantitative PCR (Fig. 6a). The tissue expression of TGF-β is markedly elevated following chronic CCL\textsubscript{4} liver injury compared with control. However, there was no significant difference in hepatic TGF-β mRNA expression between WT and Galectin-3\textsuperscript{-/-} mice in our model of liver fibrosis (Fig.6a). Furthermore, we examined TGF-β expression by ELISA in WT and Galectin-3\textsuperscript{-/-} macrophages and HSC in tissue culture and this also demonstrated no difference in levels of expression of this pro-fibrotic cytokine between the two genotypes (Fig. 6b,c). These data show that disruption of the Galectin-3 gene blocks fibrosis despite similar expression levels of TGF-β. After binding to the TGF-β receptors, TGF-β signals within the cell through the Smad family of transcriptional activators. In the presence of TGF-β ligand, the receptor-activated Smads (R-Smads), Smad2 and 3, are phosphorylated directly by the TGF-β receptor I kinase, bind to the common mediator Smad, Smad4, and translocate into the nucleus (Heldin \textit{et al.}, 1997). We therefore examined Smad2 and Smad3 phosphorylation in response to TGF-β. TGF-β stimulated a similar increase in Smad2 and Smad3 phosphorylation in WT and Galectin-3\textsuperscript{-/-} HSC (Fig. 6d). Previously (Fig. 4) we demonstrated that addition of exogenous Galectin-3, in the presence of 16% fetal calf serum, to Galectin-3\textsuperscript{-/-} HSC induced expression of α-SMA and procollagen(I). In order to address specifically the contribution of TGF-β to this observation we repeated the experiments in serum free media augmented with TGF-β (5ng/ml). The Galectin-3\textsuperscript{-/-} HSC
still exhibited a reduced activation profile as demonstrated by western blotting for \(\alpha\)-SMA (Fig. 6e) and real-time PCR quantitation of mRNA transcripts for \(\alpha\)-SMA (Fig. 6f) and procollagen(I) \((P<0.0001)\) (Fig. 6g) compared with WT HSC in the presence of TGF-\(\beta\). This defect in \textit{Galecin-3}^{-/-} HSC was overcome by the addition of exogenous recombinant Galectin-3 (Fig. 6h) with a return to activated WT morphology and \(\alpha\)-SMA filament organisation. Western blotting for \(\alpha\)-SMA (Fig. 6i) and real-time PCR quantitation of mRNA transcripts for \(\alpha\)-SMA (Fig. 6j) and procollagen(I) (Fig. 6k) \((P<0.0001)\) confirmed the morphological changes observed. No difference was seen in either \(\beta_1\) integrin expression or adhesion between WT and \textit{Galecin-3}^{-/-} HSC (data not shown). Thus, Galectin-3 is essential for TGF-\(\beta\) driven myofibroblast activation. Maeda N, \textit{et al.}, (2003) previously suggested that Galectin-3 dependent ERK1/2 activation in HSC was PKC dependent using a pharmacological inhibitor of PKC. However we demonstrated no difference in the activation of PKC and ERK1/2 in response to PDGF-BB in WT, \textit{Galecin-3}^{-/-} (and knock down with siRNA) HSC (Fig. 6l).
Figure 6

(a) TGF-β/18S expression in WT and Gal-3−/− macrophages treated with CCL4.

(b) TGF-β concentration in WT and Gal-3−/− macrophages.

(c) TGF-β concentration in WT and Gal-3−/− HSCs.
Figure 6

d

- p-Smad 2
- p-Smad 3
- β-actin

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e

- α-SMA
- β-actin

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Figure 6

f

\[ \alpha\text{-SMA/18S} \]

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+ TGF-\(\beta\)

iii

\[ \text{Procollagen(I)/18S} \]

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+ TGF-\(\beta\)

h

i

ii

iii

WT Galectin-3\(^{-/-}\) Galectin-3\(^{-/-}\) +rG-3

SF media + TGF-\(\beta\)
Figure 6

i

\[ \alpha^{-}\text{SMA} \rightarrow \text{WT} \quad \text{G-3}^{-/-} \quad \text{G-3}^{-/-} + \text{rG-3} \]

\[ \beta^{-}\text{-actin} \rightarrow \text{SF media + TGF-}\beta \]

j

\[ \alpha^{-}\text{-SMA}/18S \]

WT \quad G-3^{-/-} \quad G-3^{-/-} + \text{rG-3} 

SF Media + TGF-\beta

k

\[ \text{Procollagen(I)/18S} \]

WT \quad G-3^{-/-} \quad G-3^{-/-} + \text{rG-3} 

SF Media + TGF-\beta
Figure 6
Galectin-3 regulates myofibroblast activation and hepatic fibrosis despite similar levels of TGF-β expression and signaling.

(a) Real-time PCR quantitation of TGF-β expression in whole liver homogenates from control (olive oil vehicle) and chronic CCL4 (8 weeks) treated WT and Galectin-3−/− mice (n=6 mice in each group) (P=NS). (b) TGF-β activity measured by ELISA in supernatants recovered from mature WT and Galectin-3−/− BMDMs and (c) supernatants recovered from day four HSCs cultured for 24 hours in serum free (SF) media. (d) Smad activation. HSCs (day 4) were quiesced in serum free media for 24 hrs and stimulated with TGF-β for 60 minutes. Lysates were western blotted for phosphorylated Smad2 (pSmad2) and phosphorylated Smad3 (pSmad3). (e) Western blot analysis of α-SMA expression in HSCs (day 4) quiesced in serum free (SF) media for 24 hrs and stimulated with TGF-β (5ng/ml) for 48 hours. (f) Real time PCR quantitation of α-SMA expression and (g) procollagen(I) expression in HSCs cultured in the presence of TGF-β (5ng/ml) for 48 hours *** (P < 0.0001 compared with WT). (h) Indirect immunofluorescence for α-SMA expression (green) in HSCs (day 4) quiesced in serum free (SF) media for 24 hrs and stimulated with TGF-β or TGF-β plus recombinant Galectin-3 (30ug/ml) for 48 hours. (i) Western blot of α-SMA expression. (j) Real time PCR quantitation of α-SMA expression and (k) real time PCR quantitation of procollagen(I) expression *** (P < 0.0001 compared with WT).
siRNA-mediated inhibition of hepatic stellate cell activation in vivo.

siRNA targeted to Galectin-3 was used to establish the function of Galectin-3 in tissue fibroblast activation to myofibroblasts in vivo in our model of hepatic injury (Fig. 7). Following acute CCL4 liver injury, HSC activation peaks at day 3 and then resolves over the next four days. Galectin-3 siRNA was administered locally via the portal vein two hours after CCL4 I.P. injection. Further injections of siRNA were given hydrodynamically at 24 and 48 hours. Animals were sacrificed at 72 hours to assess Galectin-3 expression and HSC activation. CCL4 resulted in equivalent liver injury throughout all treatment groups as judged by histology, inflammatory cell infiltrate and serum ALT (results not shown). Chemically modified siRNA (siSTABLE, Dharmacon) was employed for this study as standard siRNA is rapidly degraded by nucleases present in blood and biological fluids. Efficiency of knock-down of Galectin-3 in vivo using siRNA was assessed by immunohistochemistry (Fig.7a) and real-time PCR (Fig.7b). siRNA treatment resulted in a significant decrease in Galectin-3 expression (Fig.7b). Furthermore a significant reduction in α-SMA expression was observed in Galectin-3 siRNA treated animals relative to duplex controls (Fig.7b). siRNA was well tolerated and no side-effects were noted in the treated animals. These data demonstrate that Galectin-3 siRNA can block HSC activation in response to liver injury in vivo confirming that Galectin-3 upregulation is a key mechanism in HSC activation in vivo and in the pathogenesis of liver fibrosis.
Figure 7

a

Galectin-3

PBS  |  CD  |  siRNA

α-SMA

PBS  |  CD  |  siRNA

CCL₄

b

Galectin-3/18S

PBS  |  CD  |  siRNA

α-SMA/18S

PBS  |  CD  |  siRNA

**
Figure 7

SiRNA-mediated inhibition of hepatic stellate cell activation in vivo.

(a) Galectin-3 and α-SMA staining of liver tissue harvested 3 days after CCL₄ injury (n=6 mice in each group). Mice received saline (PBS), control duplex (CD) or Galectin-3 siRNA (siRNA). Scale bar, 200 µm. (b) Left panel: Real-time PCR quantitation of Galectin-3 expression in liver homogenates 3 days after CCL₄ injury. **P<0.01 compared with control duplex. Right panel: Real-time PCR analysis of α-SMA expression in liver homogenates 3 days after CCL₄ injury. ** (P <0.01 compared with control duplex). (n=6 mice in each group).
3.5 Discussion

We have shown for the first time a central and fundamental role for Galectin-3 in the regulation of hepatic stellate cell activation in vitro and in vivo, thereby identifying Galectin-3 as a potential therapeutic target in the treatment of liver fibrosis. Our data indicate: 1) Galectin-3 expression is upregulated in established human fibrotic liver disease, during the development of experimental liver fibrosis and is temporally and spatially related to the induction and resolution of experimental hepatic fibrosis. 2) Disruption of the Galectin-3 gene results in markedly reduced myofibroblast activation and liver fibrosis. 3) Ex-vivo studies of primary murine and human hepatic stellate cells revealed that Galectin-3 expression is upregulated during transition from the quiescent to the activated myofibroblastic phenotype. 4) Galectin3-/- HSC exhibited decreased proliferation and defective activation to the myofibroblast phenotype ex-vivo, an effect reversed by the addition of exogenous recombinant murine Galectin-3. 5) Inhibition of Galectin-3 expression by siRNA in WT primary murine and human HSC ex-vivo significantly reduced Galectin-3, α-SMA and procollagen(I) expression demonstrating the ability of Galectin-3 siRNA to reverse murine and human hepatic stellate cell activation. 6) TGF-β stimulated HSC activation and procollagen production requires Galectin-3. 7) In vivo siRNA knockdown of Galectin-3 inhibits HSC activation supporting the targeting of Galectin-3 as a potential anti-fibrotic therapeutic strategy.

Our human biopsy data demonstrates that Galectin-3 expression is increased in human liver fibrosis secondary to diverse types of injury, ranging from viral-mediated (Hepatitis B and C) to metabolic disease (iron overload). This suggests that Galectin-3 upregulation is a basic response within the liver regardless of the initiating agent or disease process. The pattern of Galectin-3 staining observed in the human tissue was different from the distribution observed in our animal models. This difference relates to the chronicity and intensity of the disease processes. Human tissue was taken from patients with advanced cirrhosis following years (in many cases decades) of chronic injury and fibrosis. In our mouse and rat models a much shorter time-course of injury (8 and 12 weeks respectively) leading to liver fibrosis was examined. It seems likely that if the animal models were
allowed to run on for years, a similar pattern of fibrosis and Galectin-3 expression would be observed.

In our experimental model of liver fibrosis, there was a very close spatial and temporal relationship between Galectin-3 expression, myofibroblast activation and collagen deposition. Galectin-3 can be considered an immediate early gene and is upregulated rapidly in response to tissue injury (Kadrofske et al., 1998; Chiariotti et al., 2004). Our results demonstrated that spontaneous HSC activation occurs in WT but not Galectin-3/- HSC and that this defect can be overcome by exogenous addition of Galectin-3 (which is rapidly internalised by HSC). Spontaneous activation of WT HSC was blocked by siRNA knockdown of Galectin-3 expression. These results suggest that Galectin-3 autocrine stimulation of HSC is sufficient for HSC activation in vitro. However, the peri-sinusoidal orientation and long cytoplasmic processes of HSC facilitate their interactions with neighboring cell types including other non-parenchymal cells such as kupffer cells and sinusoidal endothelial cells and liver parenchymal cells (hepatocytes). This may regulate HSC phenotype and function by facilitating both autocrine and paracrine activation of myofibroblasts by Galectin-3 via cell-cell contacts, cell-matrix contacts and via soluble factors. Furthermore, within the injured liver, injured epithelium (hepatocytes) upregulate Galectin-3 expression following injury and both recruited and resident tissue macrophages are abundant sources of Galectin-3 (Sato and Hughes, 1994). Thus both autocrine and paracrine Galectin-3 stimulated HSC activation may exist during liver inflammation and fibrosis in vivo.

TGF-β is a major pro-fibrogenic cytokine and is a key mediator of fibrosis in many different organs (Leask and Abraham, 2004). TGF-β mRNA expression was markedly elevated following hepatic injury, however expression of TGF-β was similar in whole liver homogenates from fibrotic liver in WT and Galectin-3/- mice. Secretion of TGF-β was the same in WT and Galectin-3/- macrophages and HSC, and Smad-2 and Smad-3 signaling in HSC was similar between the two genotypes when stimulated with TGF-β. However, despite similar levels of TGF-β and intact TGF-β signaling pathways, the
absence of Galectin-3 markedly inhibited the fibrotic phenotype \textit{in vitro} and \textit{in vivo} in our animal model. This demonstrates that TGF-β stimulated HSC activation and procollagen production requires Galectin-3.

Galectin-3 can form pentamers in the presence of multivalent ligands, cross-linking glycoproteins at the cell membrane (Ahmad et al., 2004). The resultant superstructure of galectins and glycoproteins at the cell surface can bind cell-surface receptors such as the epidermal growth factor receptor (Partridge et al., 2004) regulating receptor activation and intracellular signalling. Our time-course experiments examining trafficking of exogenous recombinant Galectin-3 added to primary HSC suggest that Galectin-3 is rapidly internalised. Furthermore siRNA mediated knockdown of Galectin-3 inhibited myofibroblast activation and procollagen expression. Intracellularly, Galectin-3 can shuttle between the nucleus and the cytoplasm (Davidson et al., 2002) and is involved in fundamental processes such as pre-mRNA splicing (Dagher et al., 1995; Wang JL et al., 2004), cell-cycle progression (Kim et al., 1999; Lin et al., 2002), proliferation (Moutsatsos et al., 1987; Inohara et al., 1998; Shimura et al., 2004), and apoptosis (Yang et al., 1996; Akahani et al., 1997; Honjo et al., 2001; Yu et al., 2002) mainly through intracellular protein-protein interactions rather than lectin-carbohydrate interactions. However the precise mechanisms by which Galectin-3 regulates these intracellular processes have still to be defined. Recently it has been shown that TGF-β can induce renal fibrosis in a Smad2/3-independent fashion (Moustakas and Heldin, 2005; Wang et al., 2005) and activates additional signaling molecules such as p38 (Tsukada et al., 2005), bcr-abl (Wang et al., 2005) and PAK2 (Wilkes et al., 2003). Our results suggest that TGF-β requires intracellular Galectin-3 to stimulate myofibroblast activation and procollagen production independent of Smad-2 and Smad-3.

RNA interference allows in-depth study of the molecular mechanisms of disease through specific gene target inhibition. Furthermore, siRNAs hold direct therapeutic promise, as agents capable of attenuating the expression of disease-causing genes (Soutschek et al., 2004). We used siRNA duplexes to specifically examine the role of Galectin-3 in
myofibroblast activation and liver fibrosis \textit{in vitro} and \textit{in vivo}. SiRNA silencing of Galectin-3 expression in both primary mouse and human HSC resulted in inhibition of myofibroblast activation and procollagen (I) expression. Multiple duplexes were employed for silencing experiments to ensure that target knockdown correlated with the observed functional outcomes. siRNA knockdown of Galectin-3 \textit{in vivo} reduced myofibroblast activation in our model of hepatic injury. Thus Galectin-3 is critical for myofibroblast activation \textit{in vivo}. Strategies to knockdown expression of Galectin-3 in the liver may lead to the development of novel anti-fibrotic therapies.
CHAPTER 4

CD98hc (SLC3A2) interaction with β1 integrins is required for transformation.

Molecular mechanisms of cellular transformation

Within tissues acute inflammation can evolve into chronic inflammation with the formation of fibrous scar tissue, and a resultant increased predisposition to malignant transformation. Many cancers show abnormalities of integrin function as a result of transformation by oncogenes (Zou et al., 2002). More importantly, the growth of several tumours depends on β1 integrin function (Weaver et al., 1997). Galectin-3 has been identified as a ligand for the transmembrane protein CD98 (an early T-cell activation antigen) (Dong and Hughes, 1997). CD98 has been identified as a central activator of integrin function (Fenczik et al., 1997). Galectin-3 was further shown to be an endogenous cross-linker of the CD98 antigen, resulting in the activation of integrin mediated adhesion suggesting that Galectin-3 could promote CD98 dimerization and, indirectly, integrin activation (Hughes, 2001). Previous work demonstrated that cross-linking CD98 (with the 4F2 monoclonal antibody) promoted integrin-like signaling and anchorage-independent growth (Rintoul et al., 2002). However, the mechanism by which CD98hc associates with and regulates integrin function and what role this plays in transformation is unclear. The third aim of this thesis was to investigate the mechanism by which overexpression of CD98hc (a ligand for Galectin-3) leads to cellular transformation, in particular assessing the relationship between transformation, PI3K activation, and β1 integrins.
4.1 Abstract

CD98hc (SLC3A2) constitutively and specifically associates with β1 integrins and is highly expressed on the surface of human tumor cells irrespective of the tissue of origin. We have found here that expression of CD98hc promotes both anchorage- and serum-independent growth. This oncogenic activity is dependent on β1 integrin-mediated phosphoinositol 3-hydroxykinase stimulation and the level of surface expression of CD98hc. Using chimeras of CD98hc and the type II membrane protein CD69, we show that the transmembrane domain of CD98hc is necessary and sufficient for integrin association in cells. Furthermore, CD98hc/β1 integrin association is required for focal adhesion kinase-dependent phosphoinositol 3-hydroxykinase activation and cellular transformation. Amino acids 82–87 in the putative cytoplasmic/transmembrane region appear to be critical for the oncogenic potential of CD98hc and provide a novel mechanism for tumor promotion by integrins. These results explain how high expression of CD98hc in human cancers contributes to transformation; furthermore, the transmembrane association of CD98hc and β1 integrins may provide a new target for cancer therapy.
4.2 Introduction

The CD98 family is composed of widely expressed cell-surface disulfide-linked 125-kDa heterodimeric membrane glycoproteins containing a common glycosylated 80-kDa heavy chain (CD98hc, 4F2hc, SLC3A2) and a group of ~45-kDa light chains. Early studies of peripheral blood T lymphocytes implicated CD98hc in the regulation of cellular activation (Haynes et al., 1981). Although expressed at low levels on the surface of quiescent cells, CD98hc is rapidly up-regulated early in transition from G₀ to G₁ phase following cellular activation and remains at elevated levels until the cell cycle is complete (Azzarone et al., 1985; Suomalainen, 1986; Parmacek et al., 1989). All embryonic fibroblasts express CD98hc, and expression gradually diminishes on cells with maturity. CD98hc is highly expressed on the surface of tumor cells, irrespective of the tissue of origin (Bellone et al., 1989; Dixon et al., 1990). Deletion of CD98hc in embryonic stem cells blocks their ability to form teratocarcinomas in mice (Feral et al., 2005), and overexpression of CD98hc in murine fibroblasts results in anchorage-independent growth (Hara et al., 1999). In addition, increased CD98hc expression correlates with the development, progression, and metastatic potential of tumors (Esteban et al., 1990, Garber et al., 2001; Yoon et al., 2003). Thus, CD98hc plays an important role in tumorigenesis; however, its mechanism of action has not been determined.

The integrin family of cell-surface heterodimeric glycoproteins composed of α and β subunits function primarily as receptors for extracellular matrix ligands, which regulate many aspects of cell physiology, including morphology, adhesion, migration, proliferation, and differentiation (Schwartz, 1997). Many cancers show abnormalities of integrin function as a result of transformation by oncogenes (Zou et al., 2002). More importantly, the growth of several tumors depends on β₁ integrin function (Weaver et al., 1997). CD98hc constitutively and specifically associates with β₁ integrins (Fenczik et al., 1997; Zent et al., 2000; Merlin et al., 2001; Miyamoto et al., 2003; Rintoul et al., 2002), and accumulating evidence indicates that CD98hc plays a significant role in regulating integrin-mediated functions in cancer cells. Cross-linking CD98hc promotes activation of phosphatidylinositol 3-hydroxykinase (PI3K) (Rintoul et al., 2002) and Rap1 (Suga et al.,
2001) and enhances β1 integrin-mediated cell adhesion in a number of cancer cells, including breast and small cell lung cancer (Fenczik et al., 1997; Chandrasekaran et al., 1999), and clustering of α3β1 integrin on the surface of rhabdomyosarcoma cells (Kolesnikova et al., 2001). The mechanism by which CD98hc associates with and regulates integrin function and what role this plays in transformation is unclear.

The extracellular domain of CD98hc combines with at least six different light chains to form a series of disulfide-bonded heterodimers that are involved in L-amino acid transport. The role of the light chain in CD98hc interaction with or regulation of function of β1 integrins is controversial. Mutations of cysteine residues in CD98hc that disrupt covalent association with the light chain and that reduce amino acid transport also eliminate the transforming activity of CD98hc in BALB/3T3 cells (Shishido et al., 2000) and cause loss of β1 integrin association in low density light chain membrane fractions (Kolesnikova et al., 2001). However, these mutants still bind to free β1A cytoplasmic tails (Tac-β1) in vitro and reverse Tac-β1-induced dominant integrin suppression in Chinese hamster ovary (CHO) cells (Zent et al., 2000). However, titration of CD98hc by β1 tails is not the mechanism of Tac-β1 dominant suppression (Zent et al., 2000). In contrast, other evidence suggests that the cytoplasmic/transmembrane domain of CD98hc is the critical region mediating CD98hc alteration of β1 integrin surface distribution and cytoskeletal architecture in Madin-Darby canine kidney cells and reversal of Tac-β1 dominant suppression in CHO cells (Zent et al., 2000; Merlin et al., 2001). We have shown previously that cross-linking CD98hc stimulates PI3K activity in a β1 integrin-dependent manner (Rintoul et al., 2002). The aim of this study was to investigate the mechanism by which overexpression of CD98hc leads to cellular transformation, in particular assessing the relationship between transformation, PI3K activation, and β1 integrins. We found that cellular transformation by overexpression of CD98hc depends on activation of PI3K mediated by focal adhesion kinase (FAK). This PI3K activation depends on the interaction of β1 integrins with CD98hc and is associated with redistribution of the integrins. Finally, we found that the CD98hc transmembrane domain is necessary and sufficient for integrin association and PI3K activation and transformation by CD98hc.
This protein plays an important role in the formation of certain tumours; this study defines the CD98hc interactions and resulting signaling events that lead to transformation.

4.3 Materials and Methods

DNA Constructs
Human full-length CD69 was kindly provided by Dr. F Sanchez-Madrid (Universidad Autonoma de Madrid, Madrid, Spain). The CD98hc chimeras were made by overlap PCR or restriction digestion and religation. \( \text{C}_{98}\text{T}_{69}\text{E}_{98} \) contains amino acids 1–81 of CD98hc (Swiss-Prot accession number P08195), amino acids 121–183 of CD69 (Swiss-Prot accession number Q07108), and amino acids 105–529 of CD98hc. \( \text{C}_{69}\text{T}_{98}\text{E}_{98} \) contains amino acids 1–40 of CD69 and amino acids 82–529 of CD98hc. \( \text{C}_{98}\text{T}_{98}\text{E}_{69} \) contains amino acids 1–104 of CD98hc and amino acids 62–199 of CD69. \( \text{C}_{98}\text{T}_{69}\text{E}_{69} \) contains amino acids 1–81 of CD98hc and amino acids 41–199 of CD69. \( \text{C}_{69}\text{T}_{98}\text{E}_{69} \) contains amino acids 1–40 of CD69, amino acids 1–40 82–104 of CD98hc, and amino acids 1–40 62–199 of CD69. \( \text{C}_{98}\text{T}_{69}\text{E}_{98} \) contains amino acids 1–81 of CD98hc and amino acids 41–199 of CD69. \( \text{C}_{69}\text{T}_{98}\text{E}_{69} \) contains amino acids 1–40 of CD69, amino acids 1–40 82–104 of CD98hc, and amino acids 1–40 62–199 of CD69. \( \text{C}_{69}\text{T}_{69}\text{E}_{98} \) contains amino acids 1–61 of CD69 and amino acids 105–529 of CD98hc. CD98hc(Δ2–77) has a deletion of amino acids 2–77, which removes the entire cytoplasmic domain of CD98hc, maintaining the initiator methionine as well as the presumptive stop transfer sequence Val-Arg-Thr-Arg. CD98hc(Δ1–86) (also previously termed D5; kindly provided by Drs. D. Merlin and J. L. Madara) (Merlin et al., 2001) has a deletion of amino acids 1–86, which removes the entire cytoplasmic domain and the five proximal amino acids of the predicted transmembrane domain. The above cDNAs were subcloned into pcDNA3.1 (Invitrogen), which confers neomycin resistance. The subcloned plasmids were verified by sequencing. Plasmids were purified using the QIAGEN maxi plasmid kit. cDNA encoding human LAT1, a CD98 light chain, was a kind gift from Dr. F. Verrey (University of Zurich, Zurich, Switzerland).

Antibodies
For Western blotting, the following antibodies were used: anti-protein kinase B and anti-phospho-Ser \(^{473}\) protein kinase B antibodies (New England Biolabs Inc., Beverly, MA),
anti-FAK and anti-phospho-Tyr$^{397}$ FAK antibodies (BIOSOURCE), anti-CD98 antibody (sc-7095, Santa Cruz Biotechnology), and anti-β$_1$ integrin monoclonal antibody (mAb) (141720, Transduction Laboratories). For flow cytometry, protein A-purified 4F2 was used for identification of CD98hc and CD98hc chimeras containing the extracellular domain of CD98hc. Anti-human CD69 antibody (clone FN50, Dako Corp.) was used for identification of CD69 and chimeras containing the extracellular domain of CD69. For β$_1$ integrin, rat clone 9EG7 was used (Pharmingen). For immunoprecipitation, anti-human β$_1$ integrin antibody K20 (Dako Corp.) was used. Species-specific horseradish peroxidase-labeled IgG (Dako Corp.) was used for Western blotting, fluorescein isothiocyanate-labeled secondary antibodies (Dako Corp.) for flow cytometry, and Alexa Fluor 568 and Alexa Fluor 488 (Molecular Probes, Inc.) for confocal microscopy.

**Cell Culture and Transfection**

CHO-K1 cells were obtained from the European Collection of Animal Cell Cultures and were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 1% nonessential amino acids, 5 µg/ml glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin. The cell lines GD25β$_1$ null, GD25β$_{1A}$, and GD25β$_{1A}$(Y783F/Y795F) have been described previously (Fassler et al., 1995; Sakai et al., 1998). GD25β$_1$ null cells are fibroblasts derived from β$_1$ null embryonic stem cells. The GD25β$_{1A}$ and GD25β$_{1A}$(Y783F/Y795F) mutant cell lines were derived from GD25 cells upon stable transfection with cDNAs encoding the wild-type and mutant murine β$_{1A}$ integrin subunits, respectively (Wennerberg et al., 2000). GD25β$_1$ null cells were grown in DMEM containing 10% FCS, 5 µg/ml glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin; GD25β$_{1A}$ and GD25β$_{1A}$(Y783F/Y795F) cells were grown in the same medium containing 10 µg/ml puromycin for selection. Transient transfection of cell lines with chimeric constructs was undertaken using Lipofectamine Plus (Invitrogen) following the manufacturer's instructions. Under optimal conditions, a transfection efficiency of at least 60% was achieved in each cell line. Control cells were transfected with control vector pcDNA 3.1. The hybridoma cell line 4F2 (C13) was purchased from American Type Culture Collection and cultured in DMEM containing 15% FCS, 50 units/ml...
penicillin, 50 µg/ml streptomycin, 2 mM l-glutamine, and OPI media supplement (Sigma). Secreted antibody was purified by protein G affinity chromatography.

Construction of Stable Cell Lines
Subconfluent CHO-K1 cells were transfected using Lipofectamine following the manufacturer's instructions in serum-free medium for 5 h. Serum was added for the subsequent 48 h, and transfectants were selected in medium with 1.2 mg/ml G418 (Sigma). Clones selected from each construct were maintained in 0.8 mg/ml G418 and expanded. Clones showing equivalent wild-type, chimeric, or truncated human CD98hc expression by fluorescence-activated cell sorting (FACS) analysis were selected for this study.

Flow Cytometry
Aliquots of 5 x 10^5 cells were washed and resuspended in 100 µl of phosphate-buffered saline (PBS) containing 1 µg of 4F2 (for CD98hc and chimeras containing the extracellular portion of CD98hc) or 1 µg of anti-CD69 antibody (for CD69 and chimeras containing the extracellular portion of CD69). Cells were incubated for 30 min at room temperature, followed by two washes with PBS. Samples were then incubated with species-specific fluorescein isothiocyanate-conjugated secondary antibody (1:50) for 30 min at 4 °C and again washed twice with PBS. Samples were finally resuspended in PBS and analyzed by flow cytometry using FACSCalibur (BD Biosciences). Control IgG2a and IgG1 antibodies for 4F2 and CD69, respectively, were also used.

Clonogenic Assay
Cells (2 x 10^4/ml) were suspended in 0.3% (w/v) agarose in DMEM containing 1% FCS unless indicated otherwise. The cells were layered over a solid base of 0.5% (w/v) agarose in DMEM in 6-well culture dishes. Cultures were incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. After 6 days, colonies greater than four cells were counted under a light microscope. Cloning efficiency was calculated as a percentage of the initial number of seeded cells that formed colonies.
PI3K Activity Assay

PI3K activity was measured as described previously (Moore et al., 1998). Briefly, cells were lysed in ice-cold buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM MgCl₂, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, and protease inhibitor mixture (Roche Applied Science). PI3K was immunoprecipitated from protein-equilibrated cell lysates using anti-PI3K p85α mAb (Upstate Biotechnology Inc., Lake Placid, NY) and assayed for activity using [γ-³²P]ATP and phosphatidylinositol/phosphatidylserine as substrate. 3-Phosphorylated lipids were resolved by thin layer chromatography, identified by autoradiography, and quantified by liquid scintillation counting.

Radioligand Displacement Assay for Mass Measurement of Phosphatidylinositol 3,4,5-Trisphosphate (PIP₃)

PIP₃ levels were measured as described previously (Van der Kaay et al., 1999). In brief, CHO-K1 cells (5 x 10⁶) were subjected to a standard Folch extraction, and lipid extracts containing PIP₃ were then subjected to alkaline hydrolysis, resulting in the release of the polar head group inositol 1,3,4,5-tetrakisphosphate (IP₄). The mass of IP₄ was measured by [³H]IP₄ (Amersham Biosciences) displacement from a recombinant IP₄-glutathione S-transferase-binding protein using a calibration curve obtained with unlabeled IP₄ standards.

Immunoprecipitation and Western Blotting

Confluent cultures from 100-mm plates were quiesced overnight in 0.1% FCS and washed with PBS. Cells were lysed at 4 °C in lysis buffer containing 20 mM HEPES (pH 7.4), 1% CHAPS, 150 mM NaCl, 2 mM MgCl₂, 1 mM MnCl₂, 0.5 mM CaCl₂, and EDTA-free protease inhibitor mixture (Roche Applied Science) and clarified by centrifugation for 10 min at 4 °C. Samples (20 µg of protein) were retained and solubilized in NuPAGE sample buffer (Invitrogen) for analysis of whole cell lysate by Western blotting. The remaining lysate was incubated overnight with 2 µg of immunoprecipitating antibody at 4 °C. Immune complexes were captured using 15 µl of
protein G-agarose and washed three times with lysis buffer. Following elution with NuPAGE buffer, associated proteins were resolved on 4–12% gradient gels. The proteins were transferred to nitrocellulose membranes; blocked using 3% (w/v) albumin in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.02% (v/v) Tween 20 for 1 h at room temperature; and then incubated with primary antibody overnight at 4 °C. Species-specific horseradish peroxidase-conjugated antibodies were used for secondary labeling. Immunoreactive bands were identified by enhanced chemiluminescence (Amersham Biosciences) according to the manufacturer's instructions.

**Amino Acid Transport Assay**

Cells (5 x 10^6) were washed twice and resuspended in amino acid-free and Na^+^-free uptake solution containing 100 mM choline chloride, 2 mM KCl, 1 mM MgCl_2, 1 mM CaCl_2, and 10 mM HEPES (pH 7.5). After equilibration at 37 °C for 30 min, 2 µCi of L-[4,5-3H]leucine (82 Ci/mmol) containing 2 mM unlabeled L-leucine was added to each tube, and incubation was continued for an additional 30 min at 37 °C. Cells were then placed on ice; pelleted; and washed three times with 1 ml of ice-cold wash buffer containing 80 µM choline chloride, 2 mM KCl, 1 mM MgCl_2, 1 mM CaCl_2, and 10 mM HEPES (pH 7.5). The washed cells were then digested with 200 µl of 0.2% SDS in 0.2 M NaOH for 1 h. Protein-equilibrated aliquots of 100 µl were added to scintillation fluid containing 100 µl of 0.2 M HCl, and activity was counted in a scintillation counter.

**Confocal Immunofluorescence**

Cells were plated onto glass coverslips, fixed with 3% paraformaldehyde, and quenched in 50 mM NH_4Cl. Nonspecific binding sites were blocked using 0.2% fish skin gelatin in PBS. Cells were then incubated sequentially with (i) 4F2 or fluorescein isothiocyanate-conjugated FN50, 9EG7, anti-phospho-FAK antibody, or IgG1 and IgG2A negative control antibodies and (ii) secondary Alexa Fluor antibodies. To assess the co-localization of CD98hc and β_1 integrin, incubation with 9EG7 was carried out overnight at 4 °C prior to fixation. In these experiments, incubation with 4F2 or fluorescein isothiocyanate-conjugated FN50 was performed last of all, after secondary labeling of the β_1 integrin.
Confocal microscopy was performed with a Leica TCS NT confocal microscope system, and image analysis was performed using Leica TCS NT software.

**Statistical Analysis**

Results are presented as means ± S.E. Significance of the differences between means was assessed using one-way analysis of variance (ANOVA) or two-tailed Student's $t$ test. Values of $p < 0.05$ were considered significant. Unless stated otherwise, studies were performed on three to six independent occasions.
4.4 Results

CD98hc-induced Anchorage- and Serum-independent Growth Is Dependent on the Level of CD98hc Expression and PI3K Activation

To investigate the role that CD98hc plays in cancer, we examined the effect of overexpressing human CD98hc in CHO cells on anchorage-independent growth in soft agarose, a cardinal feature of malignant transformation that closely correlates with xenograft growth in nude mice, human tumor invasiveness, and clinical aggressiveness (Bouck and Di Mayorca, 1979; Carney et al., 1980). Two clones stably expressing different cell-surface levels of CD98hc were selected. CHO cells stably expressing CD69 were used as controls. Like CD98hc, CD69 is a member of the type II transmembrane protein family. To exclude the possibility of clonal variation, three different stable clones were selected, and similar results were obtained. In addition, comparable results were obtained using a transient transfection system with transfection efficiencies >60%. Furthermore, chimeric expression did not affect β1 integrin expression as judged by flow cytometry (data not shown). The colony-forming efficiency of CHO cells stably expressing CD98hc was significantly higher than that of vector- or CD69-transfected cells. In addition, the efficiency of colony formation was greatest in the clone with the highest level of CD98hc cell-surface expression (CD98hc+) (Fig. 1A). Another feature of the transformed phenotype is the ability of cells to grow under serum-free conditions. Fig. 1B shows that overexpressing CD98hc supported anchorage-independent growth even under serum free conditions. High saturation density is also regarded as an indicator of malignant transformation. In cell culture, the CD98hc clone exhibited higher saturation density compared with CD69-transfected cells after 10 days in culture, whereas the rate of growth was unaffected (Fig. 1C).
Figure 1

A

Counts

Fluorescence

CD69

CD98hc

CD98hc (+)

B

% Cloning efficiency

CD98hc

CD69

CD98hc (+)

1% FCS

0% FCS

C

Cells x 10^5

Days

10

5

0

2

6

10

*
Figure 1

**CD98hc increases clonal growth in CHO-K1 cells.** *A*, stable transfection of CD69 or CD98hc into CHO-K1 cells. The results from FACS analysis of CD69 or CD98hc expression in the stable clones are shown (*upper panels*). Representative views from stained colonies expressing CD69 (*left panel*) and low (*middle panel*) and high (*right panel*) levels of CD98hc grown in semisolid agarose medium are shown (*lower panels*). *B*, clonal growth of CHO-K1 cells stably expressing CD98hc (*closed bars*) or CD69 (*open bars*) in semisolid agarose medium in the presence or absence of FCS. Cells (2 x 10^4/ml) were suspended in 0.3% agarose over a layer of 0.5% agarose in DMEM containing FCS as indicated. After 6 days, colonies greater than four cells were counted under a light microscope. Results are expressed as percent cloning efficiency and are the means ± S.E. of three independent experiments, *, significantly different from CD69-transfected cells (*p* < 0.05, Student's *t* test). *C*, CD98hc overexpression results in high saturation density. The growth of CD69-expressing (O) and CD98hc-expressing (■) cells in 10% FCS in standard tissue culture was measured. The results are the means ± S.E. of three independent experiments.
PI3K plays a key role in integrin activation and cellular activation and transformation (Carpenter and Cantley, 1996). We therefore examined the effect of overexpressing CD98hc on PI3K activity in CHO cells. Expression of CD98hc significantly increased PI3K activity compared with expression of CD69 by 2–2.5-fold and increased phosphorylation of protein kinase B (Fig. 2A). The PI3K inhibitor LY294002 (Vlahos et al., 1994) caused a marked concentration-dependent inhibition of the colony-forming ability of CHO cells stably overexpressing CD98hc (IC$_{50}$ = 2.1 µM) (Fig. 2B). Thus, increased expression of wild-type CD98hc acts like an oncogene, stimulating serum- and anchorage-independent clonal growth. These effects are dependent on the level of CD98hc cell-surface expression and are blocked by inhibiting PI3K activation.
Figure 2

A

PI3K Activity (c.p.m x 10^3)

<table>
<thead>
<tr>
<th>p85α</th>
<th>PIP₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD69</td>
<td>CD98hc</td>
</tr>
</tbody>
</table>

B

% Cloning efficiency

<table>
<thead>
<tr>
<th>[LY294002] μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>30</td>
</tr>
</tbody>
</table>

% Cloning efficiency vs [LY294002] μM
**Figure 2**

**CD98hc activates PI3K.** *A*, $5 \times 10^6$ CHO-K1 cells stably expressing CD69 or CD98hc (low and high (+) expressing cells) were quiesced in 0.1% FCS overnight; lysates were equalized for protein; and PI3K activity was measured in p85α immunoprecipitates using an *in vitro* kinase assay as described in "Materials and Methods." Aliquots of the lysate were subjected to SDS-PAGE and Western-blotted for the p85α subunit of PI3K to ensure equal immunoprecipitation (*first panel*). Radiolabeled PIP₃ was resolved by thin layer chromatography, visualized by autoradiography, and quantified by liquid scintillation counting (*second panel*). Results are expressed as counts/min and represent the means ± S.E. of four independent experiments (*third panel*). An autoradiograph showing the 3-phosphorylated reaction product PIP₃ is shown for a typical experiment. Aliquots of the lysate were Western-blotted with anti-phospho-Ser⁴⁷³ protein kinase B (*P-PKB; fourth panel*) or anti-protein kinase B (*PKB; fifth panel*) antibody. *B*, shown are the effects of LY294002 on CD98hc-stimulated clonal growth. The clonal growth of CD98hc⁺ cells in semisolid 0.3% agarose medium containing 1% FCS was determined at different concentrations of LY294002 as indicated. Results are expressed as percent cloning efficiency and are the means ± S.E. of three independent experiments.
The Transmembrane Domain (Amino Acids 82–104) of CD98hc Is Necessary and Sufficient for PI3K Activation, Elevation of Intracellular PIP₃, and Colony Formation

CD98hc/CD69 chimeras (in which the extracellular, transmembrane, and cytoplasmic domains of CD98hc were exchanged with those of the type II membrane protein CD69 as shown in Fig. 3) were transfected into CHO cells to investigate the structure/function relationship of CD98hc to PI3K activation and transformation. Stable CHO cell lines were generated expressing each chimera at comparable levels as judged by flow cytometry and Western blot analysis (Fig. 4). The membrane topography of CD98hc and each of the chimeras has been established previously (Fenczik et al., 2001); the C terminus is extracellular, and the N terminus is cytoplasmic. Chimeric expression did not affect β₁ integrin expression as judged by flow cytometry (data not shown).

The effect of expressing CD98hc/CD69 chimeras on PI3K activity in CHO cells was examined both by *in vitro* kinase assay and by generation of the product PIP₃ using a radioisotope dilution assay (Van der Kaay et al., 1999). The transmembrane domain of CD98hc was necessary and sufficient to activate PI3K and to elevate intracellular PIP₃ levels (Fig. 3, A and B). In particular, the C₆₉T₉₈E₆₉ chimera (extracellular and intracellular CD69 and transmembrane CD98hc) and the truncation mutant CD98hc (Δ2–77) (in which the cytoplasmic domain (amino acids 2–77) is deleted) were both able to stimulate PI3K activation and elevation of intracellular PIP₃ levels. In contrast, the chimera C₉₈T₆₉E₉₈ (in which the transmembrane domain of CD98hc is substituted with the transmembrane domain of CD69) did not stimulate PI3K activity or elevate PIP₃ levels.

The effect of stable chimeric expression on colony formation in semisolid agarose and 1% FCS is shown in Fig. 3C. All CHO cells stably expressing the transmembrane domain of CD98hc showed cloning efficiencies comparable with those of CHO cells overexpressing wild-type CD98hc. In contrast, chimeras containing the transmembrane domain of CD69 had cloning efficiencies that were not significantly different from those of vector- or CD69-transfected cells. In particular, a CD98hc mutant with the cytoplasmic
domain deleted (CD98hc(Δ2–77)) and the chimera C69T98E69 (containing the transmembrane domain of CD98hc and the extracellular and cytoplasmic domains of CD69) both markedly enhanced colony formation when stably expressed in CHO cells (30.2 ± 6.2 and 33.2 ± 3.4, respectively) compared with CD69-expressing cells (2.2 ± 1.7, mean of four independent experiments done in triplicate, mean ± S.E., p < 0.05). However, the reciprocal chimera C98T69E98 (in which the transmembrane domain of CD98hc is replaced with the CD69 transmembrane domain) did not enhance colony formation (12.1 ± 2.1, n = 4 in triplicate, mean ± S.E., p = not significant). Similar results were achieved using a transient transfection system with transfection efficiencies >60% and three different stable clones, eliminating the possibility of clonal selection. Therefore, the transmembrane domain of CD98hc is necessary and sufficient to stimulate PI3K activity, to elevate intracellular PIP3 levels, and to promote anchorage-independent growth.
Figure 3

A

C- terminus

Extracellular

Transmembrane

Cytoplasmic

N- terminus

CD69
CD98hc
C₉₈T₉₈E₆₉
C₉₈T₉₈E₆₉
CD₉₈hcΔ(2-7₇)
C₉₈T₉₈E₆₉
CD₉₈hc

Figure 3

B

p₈₅α→
PΙP₃→

%PI3K Activity

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Figure 3

B

![Bar chart showing PIP3 mass (pmol/mg protein) for different samples.](chart_B)

C

![Bar chart showing % Cloning Efficiency for different samples.](chart_C)
Figure 3

The transmembrane domain of CD98hc (amino acids 82–104) is necessary and sufficient for PI3K activation, elevation of intracellular PIP3, and colony formation. A, the effect of chimeras on PI3K activity. Quiesced CHO-K1 cells (5 x 10^6) stably expressing CD69, CD98hc, or CD98hc chimeras were lysed, and PI3K activity was measured in p85α immunoprecipitates by in vitro kinase assay as described in "Materials and Methods." Aliquots of the lysate were subjected to SDS-PAGE and Western-blotted for the p85α subunit of PI3K to ensure equal immunoprecipitation. Radiolabeled PIP3 was resolved by thin layer chromatography, visualized by autoradiography, and quantified by liquid scintillation counting. An autoradiograph showing the 3-phosphorylated reaction product PIP3 is shown for a typical experiment. Untransfected cells stimulated with 10% FCS for 10 min or with 100 nM wortmannin for 30 min were included as controls. Results are expressed as percent activity of control untransfected cells and represent the means ± S.E. of four experiments. *, significantly different from control untransfected cells (p < 0.05, ANOVA). B, the effect of chimeras on intracellular PIP3 levels. PIP3 levels were measured in 5 x 10^6 quiesced CHO-K1 cells stably expressing CD69, CD98hc, or CD98hc chimeras. The levels of PIP3 were quantified by an isotope dilution assay as described in "Materials and Methods." Results are expressed as picomoles/mg of protein and represent the means ± S.E. of four independent experiments. *, significantly different from CD69-transfected cells (p < 0.05, ANOVA). C, the effect of CD98hc chimeras on clonal growth. CHO-K1 cells stably expressing CD69, CD98hc, or CD98hc chimeras were grown in 0.3% agarose over a layer of 0.5% agarose in 1% FCS culture medium as described in "Materials and Methods." After 6 days, colonies greater than four cells were counted by light microscopy. Results are expressed as percent cloning efficiency and are the means ± S.E. of four independent experiments. *, significantly different from CD69-transfected cells (p < 0.05, ANOVA).
Figure 4

A

![Graphs showing fluorescence counts for different proteins and variants.](image)

Fluorescence

Counts

0 10 10^3

CD69

CD98hc

C_{69}T_{98}E_{69}

C_{98}T_{69}E_{98}

C_{98}T_{98}E_{69}

CD98hc

Δ(2-77)

C_{98}T_{69}E_{69}

C_{69}T_{69}E_{98}
Figure 4

B

anti-CD69

anti-CD98

CD98hc

C₉₈T₆₉E₆₉
C₉₈T₆₉E₆₉
C₆₉T₆₉E₆₉
CD69

CD98hc

C₉₈T₆₉E₉₈
C₆₉T₆₉E₉₈
C₆₉T₆₉E₉₈
CD98hc

(Δ2-77)
Figure 4

Expression of CD98hc/CD69 chimeras in CHO-K1 cells. CD98hc/CD69 chimeras were stably transfected into CHO-K1 cells. The results from FACS analysis of expression in the stable clones using either anti-CD98 antibody 4F2 or anti-CD69 antibody are shown (A). The results from Western blot analysis of expression of CD98hc/CD69 chimeras in stable clones are also shown (B). Cell lysates were probed with anti-CD98 antibody 4F2 or anti-CD69 antibody as indicated. Analysis of expression of chimeras containing the CD69 extracellular domain was performed under nonreducing conditions, as the anti-CD69 antibody recognizes only native antigen.
The Transmembrane Domain (Amino Acids 82–104) of CD98hc Is Required and Sufficient for CD98hc and \( \beta_1 \) Integrin Co-localization

Dual label confocal immunofluorescence microscopy was used to examine the physical relationship between native \( \beta_1 \) integrins and CD98hc domains in vivo. We have shown previously that CD98hc constitutively associates with \( \beta_1 \) integrins, regardless of activation state, using stimulating, inhibitory, and neutral anti-\( \beta_1 \) integrin antibodies (Rintoul et al., 2002). As expected, CD98hc and \( \beta_1 \) integrin were co-localized in the plasma membrane (Fig. 5A). However, no co-localization was observed between CD69 (labeled by FN50) and \( \beta_1 \) integrins in CHO cells expressing CD69. To examine the biochemical basis for the interaction between CD98hc and \( \beta_1 \) integrins, the following chimeras were used: \( C_{69}T_{98}E_{69} \) (which contains the transmembrane domain of CD98hc and the extracellular and cytoplasmic domains of CD69) and CD98hc(\( \Delta 2-77 \)) (which has the cytoplasmic domain (amino acids 2–77) deleted) labeled by mAb FN50 and mAb 4F2, respectively. When stably expressed in CHO cells, both these mutants associated with \( \beta_1 \) integrins (Fig. 5A). Amaris software, which analyzes three-dimensional pixel volume co-localization, demonstrated that \( \sim 70\% \) of all \( \beta_1 \) integrins co-localized with CD98hc, \( C_{69}T_{98}E_{69} \), or CD98hc(\( \Delta 2-77 \)) (Fig. 5B). Conversely, when stably expressed in CHO cells, \( C_{98}T_{69}E_{98} \) (which contains the transmembrane domain of CD69 and the extracellular and cytoplasmic domains of CD98hc) showed less \( \beta_1 \) integrin association.
**Figure 5**

A

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B

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Figure 5
The transmembrane domain (amino acids 82–104) of CD98hc is required and sufficient for CD98hc and β1 co-localization. A, CHO-K1 cells stably expressing CD69, CD98hc, CD98hc(Δ2–77), C69T98E69, or C98T69E98 were plated onto coverslips, and the extracellular portion was labeled with mAb FN50 (CD69 and C69T98E69) or mAb 4F2 (CD98hc, C98T69E98, and CD98hc(Δ2–77)). β1 integrin was labeled with 9EG7, and immunolocalization visualized by fluorescence confocal microscopy with appropriate species-specific Alexa red (β1 integrin) and Alexa green (CD98hc/CD69) secondary antibodies. In the merged panels, areas of co-localization appear yellow. Representative cells are shown. Scale bars = 2.5 µm. B, the percentage co-localization of β1 integrin with CD69, CD98hc, CD98hc(Δ2–77), C69T98E69, or C98T69E98 was calculated using Leica TCS NT software. Results are expressed as percent co-localization and are the means ± S.E. of five independent experiments.
As an alternative approach to confocal microscopy, the association between CD98hc/CD69 chimeras and β₁ integrins was examined by co-immunoprecipitation. Stably transfected CHO cells were lysed in 1% CHAPS and immunoprecipitated with anti-β₁ integrin antibody K20. Immunoprecipitates were then separated by SDS-PAGE and Western-blotted with goat anti-CD98hc antibody. CD98hc and the CD98hc(Δ2–77) mutant (lacking the cytoplasmic domain) co-immunoprecipitated with β₁ integrins as judged by a CD98hc reactive band at 85 kDa (CD98hc) and 72 kDa (molecular mass of truncated CD98hc) (Fig. 6A). In contrast, the C₉₈T₆₆E₉₈ chimera (containing the transmembrane domain of CD69 and the extracellular and cytoplasmic domains of CD98hc) failed to co-immunoprecipitate with β₁ integrins. Thus, only those chimeras containing the CD98hc transmembrane domain co-immunoprecipitated with β₁ integrin. The reciprocal experiment was therefore carried out in which protein-equilibrated lysates were immunoprecipitated with 5 µg of anti-CD98hc mAb 4F2 or anti-CD69 mAb FN50 and subsequently blotted with anti-β₁ integrin mAb. Fig. 6B shows that, although CD69 did not immunoprecipitate with β₁ integrins, CD98hc and the chimeras containing the transmembrane domain of CD98hc did co-immunoprecipitate with β₁ integrins. By contrast, those chimeras containing the transmembrane domain of CD98hc did not, suggesting that the transmembrane domain of CD98hc is necessary and sufficient for integrin association. Thus, as judged by co-localization in situ and physical association, the transmembrane domain of CD98hc mediates its interaction with β₁ integrins. Furthermore, only those chimeras that co-localized and associated with β₁ integrins stimulated PI3K activity and anchorage-independent growth, suggesting that β₁ integrin association is required for CD98hc-mediated transformation.
Figure 6

A

anti-CD98

β₁ IP/ anti-β₁

β₁ IP / anti-CD98

CD98hc

Δ(2−77)

C₉₈T₉₈E₉₈

CD98hc(+)

CD98hc

B

CD69/CD98hc immunoprecipitation

β₁

CD69

CD98hc(+)

CD98hc

C₉₈T₉₈E₉₈

CD98hc Δ(2−77)

C₆₉T₆₉E₆₉

C₆₉T₆₉E₆₉
Figure 6

The transmembrane domain (amino acids 82–104) of CD98hc is required and sufficient for CD98hc and β1 integrin co-immunoprecipitation. A, CHO-K1 cells stably expressing CD98hc (low and high (+) expressing cells), CD98hc(Δ2–77), or C98T69E98 were lysed in 1% CHAPS immunoprecipitation buffer and adjusted to 1 mg/ml. An aliquot of the total cell lysate was resolved by SDS-PAGE and blotted with goat anti-CD98hc polyclonal antibody (upper panel). β1 integrins were immunoprecipitated (IP) from total cell lysates with 2 μg of antibody K20, and associated proteins were resolved by SDS-PAGE and blotted with anti-β1 integrin antibody mAb (middle panel) or goat anti-CD98hc polyclonal antibody (lower panel). B, cell lysates from stable chimeric clones were immunoprecipitated with 2 μg of anti-CD69 mAb FN50 (CD69, C98T69E69, and C69T98E69) or mAb 4F2 (CD98hc, CD98hc(Δ2–77), and C98T69E98). Associated proteins were resolved by SDS-PAGE, blotted with anti-β1 integrin mAb, and visualized by horse-radish peroxidase-labeled anti-mouse IgG as described in "Materials and Methods." The blots shown are representative of three independent experiments.
CD98hc Alters $\beta_1$ Integrin Surface Distribution and Promotes Extensive Focal Adhesion Complex Formation

CD98hc can influence integrin function (Fenczik et al., 1997). We therefore used confocal microscopy to examine the effect of CD98hc and chimeras on the localization of $\beta_1$ integrins and focal adhesion complexes. Expression of CD98hc altered $\beta_1$ integrin surface distribution, inducing loss of peripheral staining for $\beta_1$ integrins (Fig. 7A). Furthermore, overexpression of CD98hc promoted larger and more extensive focal adhesion complexes, consistent with increased $\beta_1$ integrin clustering (Fig. 7B). This phenotype was reproduced by the chimera $C_{69}T_{98}E_{69}$, which contains the transmembrane domain of CD98hc and the extracellular and cytoplasmic domains of CD69.
Figure 7

A

CD69

CD98hc

C_{69}T_{98}E_{69}

B

CD98hc

CD69

C_{69}T_{98}E_{69}
Figure 7

B

Quantitation of P-FAK immunostaining

Arbitrary units

CD98hc  CD69  \( C_{69} T_{98} E_{69} \)

*
Figure 7
CD98hc alters β₁ integrin surface distribution and promotes extensive focal adhesion complex formation. CHO-K1 cells expressing CD69, CD98hc, or C₆₉T₉₈E₆₉ were plated onto coverslips and fixed in paraformaldehyde. A, shown is the isosurface rendering of β₁-labeled cells expressing CD69, CD98hc, or C₆₉T₉₈E₆₉. β₁ integrin localization is shown in red on a green background. Representative cells of at least five independent experiments are shown. B, cells were stained for phospho-Tyr³⁹⁷ FAK (P-FAK) and actin (rhodamine phalloidin) and analyzed by fluorescence confocal microscopy (upper panels). Scale bars = 2 μm. Quantitation of 20 random fields/clone was performed using Openlab image analysis software (lower panel). Representative cells are shown from four independent experiments. *, significantly different from CD69-transfected cells (p < 0.05, ANOVA).
**CD98hc Signaling Is β1 Integrin- and FAK-dependent**

The protein-tyrosine kinase FAK plays a prominent role in integrin signaling. Overexpression of CD98hc caused a marked increase in FAK phosphorylation without affecting the level of FAK expression (Fig. 8A). Overexpression of the C_{69}T_{98}E_{69} chimera (extracellular and intracellular CD69 and transmembrane CD98hc) was sufficient to induce this increase in FAK phosphorylation (Fig. 8A).

GD25 cells derived from β1 null mouse embryonic endothelial cells (GD25β1 null), GD25 cells stably expressing wild-type β1 integrin (GD25β1A), and GD25 cells stably expressing a β1 integrin subunit with point mutations (GD25β1A(Y783F/Y795F), which have been shown to have a specific deficit in β1 integrin-dependent FAK activation) (Fassler et al., 1995; Sakai et al., 1998) were used to further examine the role of β1 integrins and FAK in signaling by the transmembrane domain of CD98hc. Human full-length CD98hc, CD69, and the chimera containing only the transmembrane domain of CD98hc (C_{69}T_{98}E_{69}) were transiently transfected into the GD25 cell lines. A transfection efficiency of ~50–60% was achieved in all cell lines. β1 integrin and CD98hc/CD69 expression was confirmed for each cell line using flow cytometry as described previously (data not shown) (Rintoul et al., 2002). CD98hc signaling was examined by measuring PI3K activity using an *in vitro* kinase assay. Confirmation of equal amounts of PI3K loading was obtained by probing Western blots of p85α immunoprecipitates with anti-PI3K p85α antibody (Fig. 8B). In the GD25β1A cells, overexpression of CD98hc promoted a 2.5-fold increase in PI3K activation, confirming our previous observations (Rintoul et al., 2002). The transmembrane domain of CD98hc was sufficient to induce this activity (Fig. 8B). However, overexpression of CD98hc or the C_{69}T_{98}E_{69} chimera (containing only the transmembrane domain of CD98hc) did not stimulate PI3K activity in GD25β1 null cells or GD25β1A(Y783F/Y795F) cells (Fig. 8B). Transient overexpression of CD98hc or the chimera C_{69}T_{98}E_{69} promoted a 2.5–3-fold increase in colony growth in semisolid agarose in GD25β1A cells; however, overexpression of these constructs did not stimulate clonal growth in GD25β1 null or GD25β1A(Y783F/Y795F) cells (Fig. 8C). These results show that the transmembrane interaction of CD98hc with β1
integrins is necessary for PI3K activation and anchorage-independent growth and furthermore suggest a role for FAK phosphorylation in these events.
Figure 8

A

\( \text{P}-\text{FAK} \)

\( \text{FAK} \)

CD69  CD98hc  \( C_{69}T_{98}E_{69} \)

120

B

\( \text{p85}^{\alpha} \)

\( \text{PIP}_3 \)

\( \text{PI3-kinase activity (cpm} \times 10^3) \)

\( \beta_1 \text{ integrin} \)  \( \beta_1 \text{ null} \)  \( \beta_{1A}Y783/795F \)

\( \text{CD69} \)  \( \text{CD98hc} \)  \( C_{69}T_{98}E_{69} \)
Figure 8

C

Cloning efficiency %

β₁ integrin  β₁ null  β₁AY783/795F

CD69  CD98hc  C₆₀T₉₃E₆₉
Figure 8

Signaling by the transmembrane domain (amino acids 82–104) of CD98hc is β₁ integrin- and FAK-dependent. A, CHO-K1 cells stably expressing CD69, CD98hc, or C₆₉T₉₈E₆₉ were lysed and resolved by SDS-PAGE. Blots were probed with anti-phospho-Thr³⁹⁷ FAK polyclonal antibody (P-FAK; upper panel) or anti-FAK mAb (lower panel). Bands were visualized by horseradish peroxidase-labeled secondary antibodies as described in "Materials and Methods." Representative blots of three independent experiments are shown. B, GD25 cells expressing mouse wild-type β₁ integrin, GD25β₁ null cells, or GD25β₁A(Y783F/Y795F) cells were transiently transfected with CD69 (open bars), CD98hc (closed bars), or C₆₉T₉₈E₆₉ (hatched bars) and lysed, and PI3K activity was measured as described in "Materials and Methods." Aliquots of the lysate were subjected to SDS-PAGE and blotted for the p85α subunit of PI3K to ensure equal immunoprecipitation (upper panel). PIP₃ was resolved by thin layer chromatography, visualized by autoradiography, and quantified by liquid scintillation counting (middle panel). An autoradiograph showing the 3-phosphorylated reaction product is shown for a typical experiment. Results are expressed as PI3K activity (counts/min x 10³) and represent the means ± S.E. of four independent experiments (lower panel). C, GD25 cells expressing mouse wild-type β₁ integrin, GD25β₁ null cells, or GD25β₁A(Y783F/Y795F) cells were transiently transfected with CD69 (open bars), CD98hc (closed bars), or C₆₉T₉₈E₆₉ (hatched bars). 24 h after transfection, 1 x 10⁵ live cells were grown in 0.3% agarose over a layer of 0.5% agarose in 10% FCS culture medium as described in "Materials and Methods." After 4 days, colonies greater than four cells were counted by light microscopy. Results are expressed as percent cloning efficiency and are the means ± S.E. of three independent experiments.
To further assess the dependence of PI3K activation on FAK, CHO cells stably expressing CD69, CD98hc, or CD98hc chimeras were transiently transfected with the dominant-negative FAK-related non-kinase (FRNK) (Hauck et al., 2002). The FRNK construct completely abolished the increase in PI3K activity induced by stable expression of CD98hc or the chimera containing the transmembrane domain of CD98hc (Fig. 9), demonstrating that FAK phosphorylation is required for PI3K activation.
Figure 9
Figure 9

Inhibition of PI3K activity by FRNK. Quiesced CHO-K1 cells stably expressing CD69, CD98hc, or CD98hc chimeras were transiently transfected with vector or 5 µg of FRNK. Cells were lysed; PI3K was immunoprecipitated; and activity was measured by an *in vitro* kinase assay as described in "Materials and Methods." Aliquots of the lysate were subjected to SDS-PAGE and Western-blotted with anti-FAK mAb. The blots show expression of FRNK as a 55-kDa fragment in FRNK-transfected cells. PIP₃ was resolved by thin layer chromatography and quantified by liquid scintillation counting. Results are expressed as counts/min and represent the means ± S.E. of four independent experiments.
Amino Acids 82–86 (WALLL) of CD98hc Are Required for Integrin Association, PI3K Activation, and Anchorage-independent Growth but Not Amino Acid Transport

To further examine functional interactions of CD98hc with β1 integrins, the truncation mutant CD98hc(Δ1–86) was used. The CD98hc(Δ1–86) mutant lacks amino acids 1–86 but is well expressed in CHO cells as shown by flow cytometry and confocal microscopy (Fig. 10, A and B). Although the chimera C₆₉T₉₈E₆₉ and the truncation mutant CD98hc(Δ2–77) (which lacks the putative cytoplasmic tail, amino acids 2–77) were able to co-localize with β1 integrins, to stimulate PI3K activation, and to promote anchorage-independent growth in semisolid agarose medium (Figs. 3 and 10), the CD98hc(Δ1–86) mutant was unable to co-localize with β1 integrins, failed to stimulate PI3K activity when stably expressed in CHO cells, and did not support anchorage-independent growth in semisolid agarose medium (Fig. 10, B and C). However, this mutant was still able to functionally interact with the light chain and stimulated a 2-fold increase in isoleucine transport (Fig. 10C). These results suggest that amino acids 82–86 (WALLL) at the putative cytoplasmic tail/transmembrane domain interface are required for integrin association, PI3K activation, and transformation and show that these functions are independent of amino acid transport.
Figure 10

A

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<tr>
<th>CD69</th>
<th>CD98hc</th>
<th>C_69 T_g E_69</th>
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Counts

CD98hcΔ(1-86)

Fluorescence

Counts

0 10 10^3

B

4F2 9EG7 Merged

Merged
**Figure 10**

C

![Diagram showing bar graphs for %PI3K Activity, % Cloning efficiency, and [3H] Isoleu c.p.m./mg x 10^3 for different conditions: CD69, C_{69}T_{98}E_{69}, CD98hcΔ(1-86), C_{69}T_{98}E_{69}, CD98hcΔ(1-86).](image)
Figure 10

Amino acids 82–86 of CD98hc are required for integrin association, PI3K activation, and anchorage-independent growth but not amino acid transport. A, CD69, CD98hc, C69T98E69, or CD98hc(Δ1–86) was stably transfected into CHO-K1 cells. Expression of CD98hc(Δ1–86) was determined by flow cytometric analysis with mAb 4F2. B, CHO-K1 cells stably expressing CD98hc(Δ1–86) were plated onto coverslips. CD98hc(Δ1–86) was labeled with 4F2 (left panel), and β1 integrin was labeled with 9EG7 (middle panel). The merged image is shown in the right panel. Scale bars = 4.2 µm. C, the effect of stable CD98hc(Δ1–86) expression was compared with those of C69T98E69 and CD69 expression on PI3K activity (left panel), colony formation (middle panel), and isoleucine (IsoLeu) transport (right panel). The data represent the means ± S.E. of four independent experiments. *, significantly different from CD69-transfected cells (p < 0.05, ANOVA).
4.5 Discussion

In this study, we examined the structure/function relationship between CD98hc, integrin association, intracellular signaling, amino acid transport, and transformation (Fig.11). We have demonstrated the following. 1) The heavy chain of CD98 promotes anchorage- and serum-independent growth when overexpressed in CHO cells. This oncogenic activity is dependent on PI3K activation and the level of CD98hc cell-surface expression. 2) Using CD98hc chimeras, we have shown that the transmembrane domain of CD98hc is necessary and sufficient for overexpression-induced phosphorylation of FAK, activation of PI3K (and increased intracellular levels of PIP₃), and anchorage-independent growth. The transmembrane domain is also necessary and sufficient for integrin association. Furthermore, only those chimeras that associate with β₁ integrins stimulated PI3K activity and anchorage-independent growth. 3) Overexpression of human CD98hc alters β₁ integrin surface distribution and promotes extensive focal adhesion complex formation. The transmembrane domain of CD98hc is sufficient to induce these phenotypic changes. 4) The presence of β₁ integrin and FAK activation are necessary for the transmembrane domain of CD98hc to activate PI3K. 5) Using truncation mutants of CD98hc, we obtained results suggesting that the ability to promote transformation and integrin activation and signaling resides in the putative cytoplasmic/transmembrane domain interface (amino acids 82–86, WALLL). This interaction between CD98hc and β₁ integrins appears to be critical for the oncogenic activity of CD98hc.
Figure 11

X-linkage of CD98hc

Association with β1 integrin

Cell membrane

CD98hc

FAK

PI3-K

PIP2 → PIP3

PKB/AKT

Cell transformation (anchorage and serum independent growth)
Figure 11

Schematic representation of the relationship between CD98hc, integrin association, intracellular signaling and transformation.
CD98hc plays an important role in tumorigenesis. Deletion of CD98hc impairs the development of teratocarcinomas from embryonic stem cells, and overexpression of CD98hc in NIH3T3 cells leads to anchorage-independent growth and tumor development in athymic mice (Hara et al., 1999). Furthermore, cross-linking CD98hc promotes anchorage-independent growth in small cell lung cancer cells (Rintoul et al., 2002). The ability of cells to grow in soft agar is a feature of anchorage independence and pathognomonic of the transformed phenotype, correlating with tumorigenicity and invasiveness of human tumors (Carney et al., 1980). Full oncogenic transformation is believed to require both serum- and anchorage-independent growth (Schwartz, 1997). We have shown here that CD98hc-transfected CHO cells are capable of both anchorage- and serum-independent growth, suggesting that overexpression of CD98hc mediates oncogenic transformation by providing signals that normally emanate from both integrins and growth factor receptors.

β1 integrins play a central role in cancer (Weaver et al., 1997; White et al., 2001). CD98hc physically associates with β1 integrins (Zent et al., 2000; Merlin et al., 2001; Miyamoto et al., 2003), and we have shown previously that cross-linking CD98hc results in "integrin-like" signaling (Rintoul et al., 2002). However, other groups have been unable to chemically cross-link CD98hc directly to β1 integrins; hence, the interaction may not be direct (Kolesnikova et al., 2001). Nevertheless, an abundance of evidence points to the functional relevance of the CD98hc-β1 integrin complex. In vitro binding data show that the cytoplasmic and transmembrane domains of CD98hc are both necessary and sufficient for binding to free β1A cytoplasmic tails and for the reversal of dominant suppression (Fenczik et al., 2001). The results presented here show that only those chimeras that bind to β1 integrins promote integrin signaling (as evidenced by FAK phosphorylation, PI3K activation, and increased intracellular levels of PIP3) and transformation. Chimeras in which the putative transmembrane domain of CD98hc is replaced with that of CD69 lost the capacity to associate and co-localize with β1 integrins, to stimulate PI3K, and to promote anchorage-independent growth. Results obtained with truncation mutants of CD98hc suggest that amino acids 82–86 of the putative CD98hc transmembrane/membrane proximal region of the cytoplasmic domain are required for
this activity. Although amino acids 82–86 may mediate CD98/β1 integrin interactions, it is also possible that loss of these amino acids may result in a conformational change in the truncation mutant, disrupting CD98hc/β1 integrin association. Nonetheless, results obtained with truncation mutants CD98hc(Δ2–77) and CD98hc(Δ1–86) suggest the importance of the CD98hc transmembrane/membrane proximal region of the cytoplasmic domain in regulating β1 integrin function and transformation. Other integrin-binding proteins such as cytohesin-1, Rack-1, and skelemin also bind the membrane proximal region (Liu et al., 2000) and might contribute to the CD98hc/β1A tail interactions. CD98hc may also act as a "molecular facilitator" in the plasma membrane, regulating the association of integrins in the plane of the membrane with transmembrane domains from the superfamily members CD81, CD82, CD63, and CD53, modifying the positive or negative regulatory effects of these proteins on integrin activity.

CD98hc expression is high on the surface of human tumor cells (Bellone et al., 1989; Dixon et al., 1990). However, in untransformed cells, the cyclical endogenous expression of CD98hc does not result in the display of malignant phenotypes. Hara et al. (1999) have previously shown that increasing levels of CD98hc in NIH3T3 cells result in increased tumorigenicity. In this study, we have demonstrated that increasing levels of CD98hc cause increased levels of CD98hc-β1 integrin complexes with subsequent FAK phosphorylation, PI3K activation, and transformation. FAK is up-regulated in a wide variety of human epithelial cancers, with expression closely correlated to invasive potential. Furthermore, recent evidence demonstrated a direct link between FAK expression and tumor development in vivo and has therefore stimulated interest in strategies to block FAK function as a therapeutic intervention in cancer (McLean et al., 2003; Ruoslahti, 1999). Similarly, constitutively active PI3K can transform chick embryonic fibroblasts (Chang et al., 1997), and a mutant p85 regulatory subunit of PI3K can transform fibroblasts in vitro (Jimenez et al., 1998). In addition, PI3K acting through protein kinase B has been shown to promote anchorage-independent growth (Moore et al., 1998; Khwaja et al., 1997). Therefore, our results suggest that a major mechanism by which CD98hc overexpression drives transformation in human cancers is via an increase in CD98hc-β1 integrin complexes with subsequent β1 integrin-dependent FAK
phosphorylation and PI3K activation. The precise role of FAK in CD98hc-mediated transformation remains to be fully elucidated. FAK null cells and cells expressing dominant-negative FAK constructs have inefficient spreading or focal adhesion formation. Furthermore, confocal microscopy experiments in our laboratory (data not shown) confirm published results (Sakai et al., 1998) that, although all three GD25 cell lines spread well on fibronectin and display focal adhesion contacts and F actin, qualitative differences in F actin and focal adhesion contacts are observed. GD25β1A cells have coarser and more heterogeneous focal contacts than GD25β1 null cells, whereas GD25β1A(Y783F/Y795F) cells have finer and more uniform focal contacts. Thus, although our results suggest that FAK phosphorylation plays a direct role in CD98hc/β1 integrin-mediated PI3K activation and transformation, it remains possible that blocking integrin-mediated FAK activation blocks PI3K activation and transformation indirectly as a result of qualitative differences in spreading or focal adhesion formation.

The physical interaction of CD98hc with amino acid transporter light chains has been proposed to regulate integrins (Kolesnikova et al., 2001). CD98hc also regulates γ+L- and L-type amino acid transport (Nakamura et al., 1999; Kanai et al., 1998). This regulation appears to be due to disulfide-bonded heterodimer formation with a variety of light chains (Nakamura et al., 1999; Mastroberardino et al., 1998). In addition to covalent association, there is also a noncovalent interaction between the heavy and light chains of CD98 (Pfeiffer et al., 1998). We used CD98hc chimeras lacking the CD98hc extracellular domain to completely eliminate the possibility of interactions with the light chain. Our results show that integrin association, PI3K activation, and stimulation of anchorage-independent growth by CD98hc are functions distinct and separable from the regulation of amino acid transport. Chimeras in which the transmembrane domain of CD98hc is replaced with that of CD69 lost the capacity to associate and co-localize with β1 integrins, to stimulate PI3K, and to promote anchorage-independent growth. In contrast, these replacements had no significant effect on the amino acid transport function of CD98 (data not shown) (Zent et al., 2000). Exchange of the extracellular domain of CD98hc with that of CD69 resulted in a protein that was still capable of affecting integrin function but did not stimulate isoleucine transport. These results suggest that the association of CD98hc
with a light chain is not required for its interaction with integrins or for the functional regulation of integrins. The C109S CD98hc mutant, which blocks disulfide linkage to the light chain, has been reported to decrease integrin association (Kolesnikova et al., 2001). However, as shown here, replacement of the entire extracellular domain of CD98hc leaves this function intact. These data suggest that the effect of the C109S mutant on these functions cannot be ascribed simply to loss of light chain interaction and may result from a change in the conformation or glycosylation of CD98hc. This hypothesis is supported by the marked antigenic change in CD98hc caused by the C103S mutant (Shishido et al., 2000).

CD98hc is highly expressed on the surface of tumor cells irrespective of the tissue of origin (Bellone et al., 1989; Dixon et al., 1990). Many cancer cells show abnormalities of integrin function as a result of transformation by oncogenes (Zou et al., 2002). CD98hc modulates integrin function in cancer cells. Cross-linking CD98hc stimulates integrin α3β1-dependent adhesion in small cell lung cancer cells and certain breast cancer cell lines (Fenczik et al., 1997; Chandrasekaran et al., 1999). Our results suggest that the physical interaction between the transmembrane/membrane proximal cytoplasmic domain of CD98hc and β1 integrins leads directly to activation of the full program of integrin stimulation. This induces receptor clustering stimulating the phosphorylation of FAK, resulting in PI3K activation and transformation. No tumor-associated point mutations have been reported in CD98hc to date; however, mutations that promote its interaction with integrins would be anticipated to promote tumorigenesis. Furthermore, increased expression of CD98hc promotes oncogenesis. Similarly, overexpression of wild-type epidermal growth factor receptor family members by gene amplification or increased transcription (Plowman et al., 1993) has been implicated in a wide variety of human tumors. Receptor oncogenes such as members of the ERBB/epidermal growth factor receptor family are overexpressed in some tumor types. In contrast, there is increased expression of CD98hc on almost all tumor cells (Bellone et al., 1989; Dixon et al., 1990). Therefore, the analysis of CD98hc-mediated transformation may reveal general mechanisms involved in the oncogenic process and may provide a novel target for cancer therapy.
CONCLUDING REMARKS AND POTENTIAL FUTURE STUDIES

In this thesis I examined molecular mechanisms involved in acute and chronic liver injury, and also studied basic pathways mediating tumour promotion. I have shown a central role for JNK in the pathogenesis of murine paracetamol induced liver failure, thereby identifying JNK as a potentially important therapeutic target in the treatment of patients with paracetamol hepatotoxicity. Furthermore, in the studies of chronic liver injury I have identified Galectin-3 as an important regulator of myofibroblast activation and hepatic fibrosis. Finally, I demonstrated that CD98hc (SLC3A2) interaction with β1 integrins is required for transformation and that the transmembrane association of CD98hc and β1 integrins may provide a new target for cancer therapy.

Future studies

An area which warrants further study is the mechanism by which Galectin-3 switches on myofibroblast activation. Galectin-3 has a number of putative intra- and extracellular ligands listed in the literature (reviewed in Krzeslak and Lipinska, 2004), the functional significance of which in many cases has still to be determined. I have initiated a yeast 2-hybrid screen using human Galectin-3 as bait and aim to screen a human hepatic stellate cell cDNA library in an attempt to isolate novel binding partners for Galectin-3. This will hopefully shed light on where and how Galectin-3 is acting within hepatic stellate cells to drive the dramatic phenotype switch from fibroblast precursors to matrix secreting, contractile myofibroblasts.

Future studies of Galectin-3 and other lectins will undoubtedly fall under the auspices of a rapidly expanding field in biomedical research, namely glycomics (the identity of the entirety of carbohydrates in an organism is collectively referred to as the glycome). This area should specifically allow progress in the study of Galectin-3-glycoconjugate interactions rather than protein-protein interactions. This is a relatively new discipline of
biology that deals with the structure and function of oligosaccharides. Previously this field has been inhibited by inherent levels of complexity not seen in other areas of applied biology, but significant technological advances have begun to overcome these logistical hurdles. Recent advances in development of glycan arrays, synthesis of multivalent glycan ligands, bioengineering of cell-surface glycans and glycomics databases are providing new tools to identify the ligands of glycan binding proteins and to elucidate the mechanisms by which they participate in glycan binding protein function (Paulson et al., 2006). It is also hoped that these new platform technologies offer unique opportunities to exploit carbohydrates and glycoconjugates for drug discovery (Seeberger, 2005; Seeberger and Werz, 2005).

Can Galectin-3 siRNA block the development of fibrosis and cirrhosis or accelerate fibrosis resolution? Galectin-3 siRNA delivered via the portal vein, and subsequently systemically delivered, was able to inhibit myofibroblast activation following acute CCL4 mediated liver injury. Secondary to limitations of cost of siRNA and the inherent problems of repeat hydrodynamic (large volume) intravenous injections in a mouse model I was unable to assess whether repeat administration of Galectin-3 siRNA would be able to block the development of fibrosis or accelerate fibrosis resolution in a rodent model. However the use of siRNA in vivo is progressing rapidly. New generations of siRNA are already being developed with increased stability in blood and hence longer duration of effect. Furthermore siRNA delivery systems are also constantly evolving allowing much smaller (and more clinically relevant) injection volumes. As the field advances, new vectors and ways of targeting siRNA to specific areas/organs in the body are being developed, although we should take heed of the lessons learnt from the logistical problems of adenoviral delivery in the last 15 years. Therefore these advances may make possible the type of experiments mentioned above i.e. can longterm delivery of Galectin-3 siRNA abrogate the evolution of liver fibrosis or aid reversal of fibrosis when established.

JNK inhibition in vivo (using two JNK inhibitors with different mechanisms of action) markedly reduced mortality in murine paracetamol hepatotoxicity, with a significant
reduction in hepatic necrosis and apoptosis. JNKs have become prime targets for drug development in a very broad range of important clinical areas, including inflammation, diabetes, and cancer (Karin and Gallagher, 2005). During the past decade, a combination of high throughput screening, kinase-specific libraries and structure-based drug design has facilitated the discovery of selective kinase inhibitors (Manning and Davis, 2003). Furthermore, alternative peptide-based inhibitors of JNK (such as D-JNKI1 used in this project) are now being increasingly developed. The identification of allosteric modifiers rather than direct ATP competitors could lead to inhibitors of unprecedented specificity and efficacy (Bogoyevitch et al., 2004).

Both the JNK inhibitors (SP600125 and D-JNKI1) used in the studies described in chapter 2 were pan-JNK inhibitors. Therefore, although these inhibitors were highly efficacious in limiting liver injury following paracetamol poisoning, they did not shed further light on the relative importance of hepatic JNK1 or JNK2 expression in paracetamol hepatotoxicity. Furthermore, individual disruption of the JNK1 or JNK2 genes did not protect against paracetamol-induced liver injury. How JNK1 and JNK2 contribute in paracetamol induced liver failure would be an interesting area of further study, perhaps best addressed in the future with liver specific or even cell-specific (e.g. kupffer cell) conditional knockout of JNK1 and JNK2. This leads to another related important question, namely: is the beneficial effect of JNK inhibition in this model secondary to effects on the epithelium (hepatocytes) or inflammatory cells such as the kupffer cells? Further work examining hepatocytes and kupffer cells \textit{ex-vivo} may increase our understanding of where the beneficial effect of JNK inhibition \textit{in vivo} occurs. For example, JNK1\textsuperscript{-/-} and JNK2\textsuperscript{-/-} kupffer cells could be isolated and their activation profiles determined in response to various stimuli including pro-inflammatory cytokines. JNK1\textsuperscript{-/-} and JNK2\textsuperscript{-/-} hepatocyte death (including necrosis and apoptosis) could also be assessed \textit{in vitro} in response to paracetamol and/or TNF-\textalpha stimulation. Data from these experiments could yield further insights into the role of the JNKs in liver injury allowing further refinement and more specific targeting of the JNK pathway in liver disease.
Inhibition of JNK is a potentially exciting adjunct to N-acetylcysteine in the management of patients with paracetamol hepatotoxicity, particularly in the group of patients that present late after overdose or in which timing of the overdose is unclear. The JNK inhibitors did not appear to cause any adverse effects when administered to mice. However, because of the complex cross-talk within the JNK signaling cascade, as well as its cell-type and context specific effects, it is difficult to predict potential adverse events that might arise from sustained, systemic JNK pathway inhibition. For example, experience in other disease processes suggests that deletion of the JNK1 gene prevents insulin resistance (Hirosumi et al., 2002) but disrupts neuronal cytoarchitecture and initiates the pathology of Alzheimer's disease (Waetzig and Herdegen, 2005). Despite these caveats clinical trials with pharmacological JNK inhibitors are gathering pace. Celgene (NJ, USA) successfully completed a Phase I trial in healthy volunteers this year with CC-401, their lead JNK inhibitor, and are currently evaluating the clinical potential of CC-401 in acute myelogeneous leukemia (AML) in a Phase II clinical trial. With regard to the potential use of JNK inhibitors in the clinical setting of paracetamol induced acute liver failure it is interesting to consider that the total length of JNK inhibitor infusion may not need to be prolonged. In fulminant liver failure the vast majority of hepatocyte death and liver injury is within the first 24-36 hours. Therefore a brief course of JNK inhibitor therapy (perhaps 24-48 hours) in addition to NAC may alter clinical outcome, and would potentially abrogate concerns regarding sustained, long-term systemic JNK inhibition. In the coming years the utility of targeting the JNK pathway for therapeutic benefit in a broad range of medical conditions, including liver disease, will hopefully be determined through rigorous, randomised controlled clinical trials.
REFERENCES:


Carney DN, Gazdar AF, Minna JD: Positive correlation between histological tumor involvement and generation of tumor cell colonies in agarose in specimens taken directly from patients with small-cell carcinoma of the lung. Cancer Res 1980, 40:1820-1823


Davis RJ: Signal transduction by the JNK group of MAP kinases. Cell 2000, 103:239-252

Dixon WT, Sikora LK, Demetrick DJ, Jerry LM: Isolation and characterization of a heterodimeric surface antigen on human melanoma cells and evidence that it is the 4F2 cell activation/proliferation molecule. Int J Cancer 1990, 45:59-68


Friedman SL: Liver fibrosis - from bench to bedside. J Hepatol 2003, 38: S38-S53


Gonen T, Grey AC, Jacobs MD, Donaldson PJ, Kistler J: MP20, the second most abundant lens membrane protein and member of the tetraspanin superfamily, joins the list of ligands of galectin-3. BMC Cell Biol 2001, 2: 17


and characterization of a new oncogene derived from the regulatory subunit of phosphoinositide 3-kinase. EMBO J 1998, 17: 743-753


Kaplowitz N: Acetaminophen hepatotoxicity: what do we know, what don't we know, and what do we do next? Hepatology 2004, 40:23-6

Karin M, Gallagher E. From JNK to pay dirt: jun kinases, their biochemistry, physiology and clinical importance. IUBMB Life. 2005 Apr-May;57(4-5):283-95.


Kolesnikova TV, Mannion BA, Berditchevski F, Hemler ME: Beta1 integrins show specific association with CD98 protein in low density membranes. BMC Biochem 2001, 2:10


Suomalainen HA: The monoclonal antibodies Trop-4 and 4F2 detect the same membrane antigen that is expressed at an early stage of lymphocyte activation and is retained on secondary lymphocytes. J Immunol 1986, 137: 422-427


Uemura M, Swenson ES, Gaca MD, Giordano FJ, Reiss M, Wells RG: Smad2 and Smad3 play different roles in rat hepatic stellate cell function and alpha-smooth muscle actin organization. Mol Biol Cell 2005, 16: 4214-4224

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Galectin-3 regulates myofibroblast activation and hepatic fibrosis


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Central to fibrogenesis and the scarring of organs is the activation of fibroblasts into matrix-secreting myofibroblasts. We demonstrate that Galectin-3 expression is up-regulated in established human fibrotic liver disease and is temporally and spatially related to the induction and resolution of experimental hepatic fibrosis. Disruption of the Galectin-3 gene blocks myofibroblast activation and procollagen (I) expression in vitro and in vivo, markedly attenuating liver fibrosis. Addition of exogenous recombinant Galectin-3 in vitro reversed this abnormality. The reduction in hepatic fibrosis observed in the Galectin-3−/− mouse occurred despite equivalent liver injury and inflammation, and similar tissue expression of TGF-β. TGF-β failed to transactivate Galectin-3−/− hepatic stellate cells, in contrast with WT hepatic stellate cells; however, TGF-β-stimulated Smad-2 and -3 activation was equivalent. These data suggest that Galectin-3 is required for TGF-β mediated myofibroblast activation and matrix production. Finally, in vivo siRNA knockdown of Galectin-3 inhibited myofibroblast activation after hepatic injury and may therefore provide an alternative therapeutic approach to the prevention and treatment of liver fibrosis.

Fibrosis represents the final common pathway of chronic tissue injury. Chronic inflammation with the formation of scar tissue, loss of tissue architecture, and organ failure is a characteristic feature of the pathogenesis of many human diseases and represents a major cause of morbidity and mortality worldwide. Currently our therapeutic repertoire is limited to immunosuppression and/or organ transplantation (1, 2). Therefore, effective alternative therapies are urgently required.

The fibroblast and myofibroblast are key cells in the initiation and perpetuation of organ scarring (3, 4). Classically, quiescent tissue fibroblasts become activated to a contractile, myofibroblast matrix-secreting phenotype. Understanding the molecular mechanisms that drive this phenotype switch may allow the development of targeted antifibrotic therapies.

Galectins are members of a growing family of animal lectins (5–8). Galectin-3 is a β-galactoside-binding animal lectin of ~30 kDa. This unique Galectin is composed of two domains: a carboxy-terminal domain that contains the carbohydrate-binding region and an amino-terminal domain consisting primarily of tandem repeats of nine amino acids (9) to cross-link carbohydrate and noncarbohydrate ligands. Galectin-3 is a pleiotropic molecule found in the nucleus, cytoplasm, and at the cell surface and can also be secreted by an unorthodox mechanism that bypasses the endoplasmic reticulum and the Golgi apparatus (10). In vitro Galectin-3 has been implicated in a variety of biological processes including cell proliferation (11, 12), adhesion (13–15), and survival (16, 17). Initial in vivo studies demonstrated that Galectin-3 knockout mice have attenuated peritoneal inflammatory responses to thioglycollate instillation (18, 19), suggesting a role for Galectin-3 in the development of acute inflammation. However, the mechanisms that are involved in fibrogenesis are distinct from those involved in inflammation (20). Increased Galectin-3 expression has been noted in tissue fibrosis (21–23), and in vitro exogenous Galectin-3 stimulates myofibroblast proliferation (24, 25). The relevance of these observations to the mechanistic role of Galectin-3 in the pathogenesis of tissue fibrosis in vivo has not been examined. We therefore examined myofibroblast activation and collagen deposition in an experimental model of hepatic fibrosis by using mutant mice lacking the Galectin-3 gene.

Results

Galectin-3 Expression Is Up-Regulated in Human Liver Fibrosis and Is Temporally and Spatially Related to Fibrosis in a Reversible Rat Model of Liver Fibrosis. In established human liver fibrosis, regardless of etiology (hepatitis B or C, autoimmune, copper or iron overload, primary biliary cirrhosis, or alcohol-induced), Galectin-3 expression was negligible in normal liver and dramatically increased in the cirrhotic nodules of hepatocytes, particularly at the periphery of the nodules (n = 36 cases) (Fig. 1a and b). Furthermore, Galectin-3 expression was examined in a well established rat model of reversible carbon tetrachloride (CCL4)-induced liver fibrosis (26, 27) (Fig. 1c). Galectin-3 expression was temporally and spatially associated with fibrosis [collagen fibers stained red with picrosirius red (PSR)], minimal in normal rat liver, maximal at peak fibrosis, and was virtually absent again at 24 weeks (recovery from fibrosis). This finding suggests that the development (and resolution) of fibrosis may be regulated by Galectin-3.

Galectin-3 Plays a Critical Role in Liver Fibrosis. The significance of the above observations was examined by using CCL4-induced liver injury as a model system for liver fibrogenesis. After 8 weeks of CCL4 treatment, increased Galectin-3 expression was observed in the perportal areas and areas of bridging fibrosis in the liver. Dense Galectin-3 staining was noted at the periphery of the inflamed damaged areas. Galectin-3 was only expressed in bile duct epithelia and Kupffer cells in the liver from control (olive oil treated) animals (Fig. 2a). A marked increase in Galectin-3 mRNA (real-time PCR) and protein expression (Western blot analysis) was demonstrated with the development of hepatic fibrosis in the CCL4-treated animals compared with control (Fig. 2b). Hepatic collagen was stained with PSR (Fig. 2c) and quantified by using digital image analysis (Fig. 2d Left). Striking collagen deposition in the same distribution as Galectin-3 was present in the perportal areas and areas of bridging fibrosis in the WT animals. By contrast, there was significantly less collagen deposition in the Galectin-3−/− mice (P < 0.05). Procollagen (I) mRNA expression [collagen (I) is highly expressed in human and animal models of liver fibrosis] was significantly decreased in the livers from Galectin-3−/− compared with WT mice after chronic CCL4 treatment as judged by real-time PCR (P < 0.05) (Fig. 2d Right).

Conflict of interest statement: No conflicts declared.

Abbreviations: α-SMA, α-smooth muscle actin; CCL4, carbon tetrachloride; HSC, hepatic stellate cell; PSR, picrosirius red.

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Disruption of the Galectin-3 Gene Does Not Affect Initial Liver Injury or Inflammatory Cell Infiltrate After CCL4 Treatment. After a single i.p. injection of CCL4, initial hepatic injury as judged by alanine aminotransferase (a marker of hepatocyte damage) was similar in WT and Galectin-3−/− mice at days 1–7 (Fig. 6a and b, which is published as supporting information on the PNAS web site). Furthermore, total liver tissue levels of the proinflammatory cyto-

contractile myofibroblasts with increased expression of α-smooth muscle actin (α-SMA), a widely accepted marker of myofibroblast activation (3, 4) in vitro and in vivo. After 8 weeks of CCL4 administration, α-SMA expression was markedly increased in WT compared with Galectin-3−/− mice with the same temporal and spatial distribution as Galectin-3 and collagen expression (Fig. 2e). There was significantly less α-SMA expression in the Galectin-3−/− mice compared with WT quantified by using digital image analysis (Fig. 2f Left; P < 0.01). The transcripts for α-SMA mRNA, as assessed by real-time PCR, were significantly increased in WT animals after CCL4 treatment compared with animals that received olive oil (control) (Fig. 2f Right). However α-SMA transcripts were significantly decreased in Galectin-3−/− mice compared with WT (P < 0.01). This decrease in mRNA expression was paralleled by a decrease in hepatic α-SMA protein expression assessed by Western blot analysis of whole liver tissue (Fig. 2f Right Inset). These data indicate that Galectin-3 mediated activation of HSCs in vivo is a central mechanism underlying hepatic fibrosis. Furthermore, we investigated myofibroblast activation in models of renal fibrosis and pulmonary fibrosis by using unilateral ureteric obstruction and intratracheal instillation of silica respectively (N.C.H. and T.S., unpublished observations). In both instances, we found defective myofibroblast activation in the Galectin-3−/− mice. This finding demonstrates the broad applicability of our results and potential relevance to many forms of tissue fibrosis in different disease states.

Right), suggesting that Galectin-3 regulates hepatic collagen deposition during liver injury. We therefore went on to examine the mechanism underlying this important observation.

The hepatic stellate cell (HSC) is the key fibrogenic cell of the liver and represents a paradigm cell type in studies of the pathogenesis of tissue fibrosis (3, 4, 28). After any cause of liver injury, quiescent HSCs undergo activation to proliferative, fibrogenic, and

Fig. 1. Galectin-3 expression is up-regulated in human liver fibrosis. (a) Galectin-3 expression in normal human liver. (b) Galectin-3 in cirrhosis secondary to hepatitis C infection. (Scale bar: 400 μm.) Galectin-3 expression is temporally and spatially related to fibrosis in a reversible rat model of liver fibrosis. (c Upper) Collagen stained with PSR. (Scale bar: 100 μm.) (c Lower) Galectin-3 immunohistochemistry. (Scale bar: 200 μm.) (Left) Control (olive oil vehicle only). (Middle) Peak fibrosis in rat liver after 12 weeks of twice weekly i.p. CCL4. (Right) Resolution, 24 weeks after cessation of CCL4-induced liver injury.

Fig. 2. Galectin-3 plays a critical role in organ fibrosis. Mice were treated with olive oil (control) or CCL4 i.p. twice weekly for 8 weeks (n = 6 mice in each group). (a) Galectin-3 expression in control (Left) and after chronic CCL4 treatment (Right) in WT mouse liver. (Scale bar: 400 μm.) (b Left) Real-time PCR quantitation of Galectin-3 expression in whole liver homogenates from control (olive oil vehicle) and chronic CCL4 treated mice. ***; P < 0.0001 compared with control. (b Right) Representative Galectin-3 and β-actin Western blots of whole liver from control and CCL4-treated WT mice. (c) Collagen staining with PSR of liver tissue after chronic CCL4 treatment of WT and Galectin-3−/− mice. (Scale bar: 200 μm.) (d Left) Digital image analysis quantification of collagen staining. *; P < 0.05. (d Right) Real-time PCR quantification of procollagen I mRNA in whole liver homogenates from chronic CCL4 and control groups. ***; P < 0.05 compared with WT. (e) α-SMA staining of liver tissue after chronic CCL4 treatment. (Scale bar: 200 μm.) (f Left) Digital image analysis quantification of α-SMA staining. ***; P < 0.01 compared with WT. (f Right) Real-time PCR quantification of α-SMA in whole liver homogenates in chronic CCL4 and control groups. **; P < 0.05 compared with WT. (f inset) Representative Western blots of α-SMA and β-actin expression in whole liver homogenates from chronic CCL4-treated mice.
kinase TNF-α in WT and Galectin-3−/− mice measured by ELISA were not significantly different 24 h post-CCL4 liver injury (WT, 1.4 ± 0.22 ng TNF-α per μg whole liver protein; Galectin-3−/−, 1.65 ± 0.3 ng TNF-α per μg whole liver protein (P value was not significant)). Hepatic inflammatory cell recruitment was similar in WT and Galectin-3−/− mice at days 1–7 (Fig. 6 c and d). Furthermore, there was no difference in T lymphocyte recruitment between WT and Galectin-3−/− mice as assessed by cluster of differentiation molecule 3 (CD3) immunohistochemistry and counting.

The macrophage in particular is an important inflammatory cell involved in the pathogenesis of tissue fibrosis (29, 30) and therefore we examined proinflammatory cytokine release from WT and Galectin-3−/− bone marrow-derived macrophages (BMDMs) (see Supporting Materials and Methods, which is published as supporting information on the PNAS web site). Maturation of WT and Galectin-3−/− BMDMs demonstrated equivalent expression of CD11b and F4/80 (Fig. 6e). After activation with IFN-γ/LPS, no significant difference was observed in the release of the proinflammatory cytokines TNF-α or IL-6 between WT and Galectin-3−/− macrophages (Fig. 6f). These data demonstrate that the difference in liver fibrosis observed between the two genotypes is not secondary to a difference in initial tissue injury, inflammatory cell recruitment, or macrophage proinflammatory cytokine release.

**Myofibroblast Activation is Galectin-3 Dependent.** HSC activation to a myofibroblast phenotype is a critical event in extracellular matrix deposition and cirrhosis (3, 4). We observed dual staining of Galectin-3 and α-SMA within cells in areas of fibrotic liver injury after 8 weeks of CCL4 treatment in WT mice. This finding demonstrates that Galectin-3 expression is up-regulated in myofibroblasts during the injury response in vivo (Fig. 3a). Galectin-3 expression was up-regulated during myofibroblast activation in vitro on tissue culture plastic in both primary murine and human HSCs (Fig. 3b). This well-established in vitro method of HSC activation closely models in vivo myofibroblast activation (26, 27) and has been used extensively to model and examine the changes that take place during the phenotype switch of fibroblasts to extracellular matrix secreting contractile myofibroblasts. After 7 days in vitro culture, protein expression of α-SMA was significantly decreased in Galectin-3−/− HSCs compared with WT HSCs (Fig. 3c). Addition of exogenous recombinant murine Galectin-3 to Galectin-3−/− HSCs in vitro reversed the Galectin-3−/− phenotype resulting in increased α-SMA expression (Fig. 3d).

A rationally designed siRNA pool targeting Galectin-3 mRNA was used to inhibit Galectin-3 expression in WT primary murine HSCs. HSCs were isolated, activated on tissue culture plastic, and treated with PBS (PBS), liposome formulated nontargeted control duplex (CD), or liposome-formulated Galectin-3 siRNA. Galectin-3 siRNA treatment reduced Galectin-3 mRNA expression 10-fold compared with control siRNA or mock treatment as assessed by quantitative real-time PCR (Fig. 4a; P < 0.0001). siRNA knockdown of Galectin-3 in HSCs did not affect cell viability assessed by trypan blue exclusion or rates of apoptosis measured by cell morphology and counting. This marked inhibition of Galectin-3 expression resulted in a significant reduction in both α-SMA and procollagen (I) mRNA expression assessed by real-time PCR (Fig. 4 b and c, respectively). Western blot analysis confirmed almost complete inhibition of Galectin-3 expression after targeted siRNA treatment relative to control treatments (Fig. 4d Top). Western blot analysis also confirmed the reduction in α-SMA protein expression (Fig. 4d Middle). These effects were verified by using four independent murine Galectin-3 targeting siRNA duplexes (as listed in Supporting Materials and Methods). To assess the potential clinical applicability of Galectin-3 siRNA as a therapy in human fibrotic liver
Fig. 4. Galectin-3 siRNA inhibits myofibroblast activation and procollagen (I) expression in HSCs. Real-time PCR quantitation of (a) Galectin-3, (b) α-SMA, and (c) procollagen (I) expression in PBS, control duplex (CD), or Galectin-3 siRNA (250 nM) treated primary mouse HSCs. ***P < 0.0001 compared with CD. (d) Western blot analysis of Galectin-3, α-SMA, and β-actin expression in primary mouse HSCs 96 h posttransfection. (e) Western blot analysis of Galectin-3, α-SMA, and β-actin expression in primary human HSCs 96 h after treatment with CD or Galectin-3 siRNA (siRNA). (f) Real-time PCR quantitation of procollagen (I) expression in primary human HSCs 96 h after treatment with either PBS, CD, or Galectin-3 siRNA (siRNA). ***P < 0.0001 compared with CD.

Disruption of the Galectin-3 Gene Does Not Affect TGF-β Expression and Smad Signaling. TGF-β is a major profibrogenic cytokine involved in the pathogenesis of fibrosis in many different organ systems (31–33). However, mechanisms of tissue fibrosis also exist that are TGF-β independent, both in the liver and other organs (34, 35). The tissue expression of TGF-β mRNA is markedly elevated as judged by real-time quantitative PCR in whole liver samples after chronic CCL4 liver injury compared with control (Fig. 7a, which is published as supporting information on the PNAS web site). However, there was no significant difference in hepatic TGF-β mRNA expression between WT and Galectin-3−/− mice in our model of liver fibrosis (Fig. 7a). ELISA demonstrated no difference in the levels of TGF-β expression in WT and Galectin-3−/− macrophages and HSCs in tissue culture (Fig. 7 b and c). Thus disruption of the Galectin-3 gene blocks fibrosis despite similar expression levels of TGF-β. In the presence of TGF-β ligand, the receptor-activated Smad family of transcriptional activators, Smad-2 and -3, are phosphorylated directly by the TGF-β receptor I kinase (36). TGF-β stimulated a similar increase in Smad-2 and Smad-3 phosphorylation in WT and Galectin-3−/− HSCs (Fig. 7d).

Addition of exogenous Galectin-3, in the presence of 16% FCS, to Galectin-3−/− HSCs induced expression of α-SMA and procollagen (I) (Fig. 3). To address specifically the contribution of TGF-β to this observation, we repeated the experiments in serum-free media augmented with TGF-β (5 ng/ml). The Galectin-3−/− HSCs still exhibited a reduced activation profile in the presence of TGF-β compared with WT HSCs, as demonstrated by Western blotting for α-SMA (Fig. 7e) and real-time PCR quantitation of α-SMA (Fig. 7f) and procollagen (I) (P < 0.0001) (Fig. 7g). Exogenous recombinant Galectin-3 rescued this defect in Galectin-3−/− HSCs, stimulating an activated WT morphology and α-SMA filament organization (Fig. 7h). Western blotting for α-SMA (Fig. 7i) and real-time PCR quantitation of mRNA transcripts for α-SMA (Fig. 7j) and procollagen (I) (Fig. 7k) (P < 0.0001) confirmed the morphological changes observed. No difference was seen in either β1 integrin expression or adhesion between WT and Galectin-3−/− HSCs (data not shown). Thus, Galectin-3 is essential for TGF-β-driven myofibroblast activation. Maeda et al. (24) suggested that Galectin-3 dependent extracellular signal-regulated kinase 1/2 (ERK1/2) activation in HSCs was PKC dependent by using a pharmacological inhibitor of PKC. However, we demonstrated no difference in the activation of PKC and ERK1/2 in response to PDGF-BB in WT, Galectin-3−/− (and knockdown with siRNA) HSCs (Fig. 7l).

Fig. 5. Galectin-3 siRNA-mediated inhibition of HSC activation in vivo. (a) Galectin-3 and α-SMA staining of liver tissue harvested 3 days after CCL4 injury (n = 6 mice in each group). Mice received saline (PBS), control duplex (CD), or Galectin-3 siRNA (siRNA). (Scale bar: 200 μm.) (b Left) Real-time PCR quantitation of Galectin-3 expression in liver homogenates 3 days after CCL4 injury. **P < 0.01 compared with CD. (b Right) Real-time PCR analysis of α-SMA expression in liver homogenates 3 days after CCL4 injury. ***P < 0.01 compared with CD.
siRNA-treated animals relative to duplex controls (Fig. 5b) \( (P < 0.01) \). siRNA was well tolerated and no side effects were noted in the treated animals. These data demonstrate that Galectin-3 siRNA can block HSC activation in response to liver injury in vivo, confirming that Galectin-3 up-regulation is a key mechanism in HSC activation in vivo and in the pathogenesis of liver fibrosis.

Discussion

We have shown a fundamental role for Galectin-3 in the regulation of HSC activation in vitro and in vivo, thereby identifying Galectin-3 as a potential therapeutic target in the treatment of liver fibrosis. Our human biopsy data demonstrate that Galectin-3 expression is increased in human liver fibrosis secondary to diverse types of injury, ranging from viral-mediated (hepatitis B and C) to metabolic disease (iron overload). This finding suggests that Galectin-3 up-regulation is a basic response within the liver regardless of the initiating agent or disease process. The pattern of Galectin-3 staining observed in the human tissue was different from the distribution observed in our animal models. This difference relates to the chronicity and intensity of the disease processes. Human tissue was taken from patients with advanced cirrhosis after years (in many cases, decades) of chronic injury and fibrosis. In our mice and rat models, a much shorter time course of injury (8 and 12 weeks, respectively) leading to liver fibrosis was examined. It seems likely that, if the animal models were allowed to run on for years, a similar pattern of fibrosis and Galectin-3 expression would be observed.

In our experimental model of liver fibrosis, there was a very close spatial and temporal relationship between Galectin-3 expression, myofibroblast activation, and collagen deposition. Galectin-3 can be considered an immediate early gene and is up-regulated rapidly in response to tissue injury (37, 38). Our results demonstrate that spontaneous HSC activation occurs in WT but not Galectin-3-/- HSCs, and that this defect can be overcome by exogenous addition of Galectin-3 (which is rapidly internalized by HSCs). Spontaneous activation of WT HSCs was blocked by siRNA knockdown of Galectin-3 expression. These results suggest that Galectin-3 autocrine stimulation of HSCs is sufficient for HSC activation in vitro. However, the perisinusoidal orientation and long cytoplasmic processes of HSCs facilitate their interactions with neighboring cell types including other nonparenchymal cells such as Kupffer cells and sinusoidal endothelial cells and liver parenchymal cells (hepatocytes). These attributes may regulate HSC phenotype and function by facilitating both autocrine and paracrine activation of myofibroblasts by Galectin-3 by means of cell–cell contacts, cell–matrix contacts, and soluble factors. Furthermore, within the injured liver, injured epithelium (hepatocytes) up-regulate Galectin-3 expression after injury, and both recruited and resident tissue macrophages are abundant sources of Galectin-3 (39). Thus both autocrine and paracrine Galectin-3-stimulated HSC activation may exist during liver inflammation and fibrosis in vivo.

TGF-β is a major profibrogenic cytokine and is a key mediator of fibrosis in many different organs (31). TGF-β mRNA expression was markedly elevated after hepatic injury; however, expression of TGF-β was similar in whole liver homogenates from fibrotic liver in WT and Galectin-3-/- mice. Secretion of TGF-β was the same in WT and Galectin-3-/- macrophages and HSCs, and Smad-2 and Smad-3 signaling in HSCs was similar between the two genotypes when stimulated with TGF-β. However, despite similar levels of TGF-β and intact TGF-β signaling pathways, the absence of Galectin-3 markedly inhibited the fibrotic phenotype in vitro and in vivo in our animal model. These data demonstrate that TGF-β stimulated HSC activation and procollagen production requires Galectin-3.

Galectin-3 can form pentamers in the presence of multivalent ligands, cross-linking glycoproteins at the cell membrane (40). The resultant superstructure of galactins and glycoproteins at the cell surface can bind cell–surface receptors such as the epidermal growth factor receptor (41), regulating receptor activation and intracellular signaling. Our time course experiments examining trafficking of exogenous recombinant Galectin-3 added to primary HSCs suggest that Galectin-3 is rapidly internalized. Furthermore, siRNA-mediated knockdown of Galectin-3 inhibited myofibroblast activation and procollagen expression. Intracellularly, Galectin-3 can shuttle between the nucleus and the cytoplasm (42) and is involved in fundamental processes such as pre-mRNA splicing (43, 44), cell-cycle progression (45, 46), proliferation (11, 12, 47), and apoptosis (16, 17, 48, 49) mainly through intracellular protein–protein interactions rather than lectin–carbohydrate interactions. However the precise mechanisms by which Galectin-3 regulates these intracellular processes still have to be defined. Recently, it has been shown that TGF-β can induce renal fibrosis in a Smad-2/-/3-independent fashion (50, 51) and activates additional signaling molecules such as p38 (52), bcr-abl (51), and PAK2 (53). Our results suggest that TGF-β requires intracellular Galectin-3 to stimulate myofibroblast activation and procollagen production independent of Smad-2 and Smad-3.

RNA interference allows indepth study of the molecular mechanisms of disease through specific gene target inhibition. Furthermore, siRNAs hold direct therapeutic promise, as agents capable of attenuating the expression of disease-causing genes (54). We used siRNA duplexes to specifically examine the role of Galectin-3 in myofibroblast activation and liver fibrosis in vitro and in vivo. siRNA silencing of Galectin-3 expression in both primary mouse and human HSCs resulted in inhibition of myofibroblast activation and procollagen (I) expression. Multiple duplexes were used for silencing experiments to ensure that target knockdown correlated with the observed functional outcomes. siRNA knockdown of Galectin-3 in vivo reduced myofibroblast activation in our model of hepatic injury. Thus, Galectin-3 is critical for myofibroblast activation in vivo. Strategies to knockdown expression of Galectin-3 in the liver may lead to the development of alternative antifibrotic therapies.

Materials and Methods

Materials. Cytokines and recombinant mouse Galectin-3 were purchased from R & D Systems and PeproTech EC (London, U.K.). All other reagents were from Sigma-Aldrich unless otherwise stated.

Animals. Generation of Galectin-3-/- mice by gene-targeting technology has been described in ref. 18. As controls, age- and sex-matched WT littermate mice were used. All procedures were undertaken with approved license from the Animal Scientific Procedures Division of the Home Office (London, U.K.).

CCL4-Induced Liver Injury Models. Acute. After overnight fast (with free access to water) mice were injected i.p. with 1 μl/g body weight sterile CCL4 in a 1:3 ratio with olive oil or olive oil (control). Livers were harvested for analysis at 24, 48, 72, 96, and 168 h.

Chronic CCL4-induced liver fibrosis. Mice were injected i.p. with 1 μl/g body weight sterile CCL4 in a 1:3 ratio with olive oil or olive oil (control) twice weekly for 8 weeks. The rat model of CCL4-induced liver fibrosis was undertaken as described in ref. 26.

Immunohistochemistry. Paraffin-embedded sections of liver were processed for immunohistochemistry and immunofluorescence as described in refs. 27 and 29. See Supporting Materials and Methods for details of primary antibodies used. Tissue fibrosis was visualized and quantified with a PSR stain as described (26). Morphometric measurements were made on 10-μm sections stained with PSR by using OPENLAB software (Improvement, Coventry, U.K.). Forty random fields from each section were analyzed at a final magnification of ×100. Each captured field was analyzed by separation into red, green, and blue (RGB) filters, and the red area was mathematically divided by the RGB area and multiplied by 100%. This
Real-Time PCR. Total RNA from whole liver was reverse transcribed into cDNA (Applied Biosystems). See Supporting Materials and Methods for details of real-time primer sequences used.

Preparation of siRNAs and siRNA Treatment in Vitro and in Vivo. siRNAs were synthesized by using 5′-silyl-2′-tris (acetoxethyl) orthoformate method (Dharmacon Research). See Supporting Materials and Methods for details of siRNA sequences used.

In vitro. Subconfluent cultures of primary mouse and human HSCs in 6-well plates were transfected with siRNA (final concentration, 250 nM) by using oligofectamine (Invitrogen). After 96 h, cells were lysed for protein or RNA extraction. In vivo. After overnight fast (with free access to water), mice were injected i.p. with 1 mL/g body weight sterile CCL4 in a 1:3 ratio with olive oil. Animals were anesthetized 2 hours later, and a cannula was inserted into the portal vein. Fifty micrograms of Galectin-3 siRNA (Duplex no. 2, siSTABLE; Dharmacon) in 300 μL PBS was rapidly delivered via the portal vein (2 mg/kg). Before removal of the cannula, Galaspon (Johnson & Johnson, Maidenhead, U.K.) was applied to the portal vein to prevent bleeding. Further delivery of Galectin-3 siRNA at 24 and 48 h was by rapid hydrodynamic tail vein injection (50 μg of siRNA in 1 mL PBS). Control mice received PBS or siCONTROL nontargeting siRNA no. 2 (Dharmacon) by the same routes. Mice were anesthetised on day 3 (n = 6 in each group), and liver tissue was fixed in buffered formalin, whereas the remaining liver was snap-frozen for protein and mRNA analysis.

Statistical Analysis. Results are presented as mean ± SEM. Significance of the differences between means was assessed by using one-way analysis of variance (ANOVA) or two-tailed Student’s t test. Values of P < 0.05 were considered significant. Unless stated otherwise, studies were performed on three to six independent occasions.
CD98hc (SLC3A2) Interaction with \( \beta_1 \) Integrins Is Required for Transformation*

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CD98hc (SLC3A2) constitutively and specifically associates with \( \beta_1 \) integrins and is highly expressed on the surface of human tumor cells irrespective of the tissue of origin. We have found here that expression of CD98hc promotes both anchorage- and serum-independent growth. This oncogenic activity is dependent on \( \beta_1 \) integrin-mediated phosphoinositol 3-hydroxykinase stimulation and the level of surface expression of CD98hc. Using chimeras of CD98hc and the type II membrane protein CD89, we show that the transmembrane domain of CD98hc is necessary and sufficient for integrin association in cells. Furthermore, CD98hc/\( \beta_1 \) integrin association is required for focal adhesion kinase-dependent phosphoinositide 3-kinase activation and cellular transformation. Amino acids 82–87 in the putative cytoplasmic/transmembrane region appear to be critical for the oncogenic potential of CD98hc and provide a novel mechanism for tumor promotion by integrins. These results explain how high expression of CD98hc in human cancers contributes to transformation; furthermore, the transmembrane association of CD98hc and \( \beta_1 \) integrins may provide a new target for cancer therapy.

The CD98 family is composed of widely expressed cell-surface disulfide-linked 125-kDa heterodimeric membrane glycoproteins containing a common glycosylated 80-kDa heavy chain (CD98hc, 4F2hc, SLC3A2) and a group of \(-45\,\text{kDa} \) light chains. Early studies of peripheral blood T lymphocytes implicated CD98hc in the regulation of cellular activation (1). Although expressed at low levels on the surface of quiescent cells, CD98hc is rapidly up-regulated early in transition from \( \text{G}_0 \) to \( \text{G}_1 \) phase following cellular activation and remains at elevated levels until the cell cycle is complete (2–4). All embryonic fibroblasts express CD98hc, and expression gradually diminishes on cells with maturity. CD98hc is highly expressed on the surface of tumor cells, irrespective of the tissue of origin (5, 6). Deletion of CD98hc in embryonic stem cells blocks their ability to form teratomas in mice, and overexpression of CD98hc in murine fibroblasts results in anchorage-independent growth (7). In addition, increased CD98hc expression correlates with the development, progression, and metastatic potential of tumors (8–10). Thus, CD98hc plays an important role in tumorigenesis; however, its mechanism of action has not been determined.

The integrin family of cell-surface heterodimeric glycoproteins composed of \( \alpha \) and \( \beta \) subunits function primarily as receptors for extracellular matrix ligands, which regulate many aspects of cell physiology, including morphology, adhesion, migration, proliferation, and differentiation (11). Many cancers show abnormalities of integrin function as a result of transformation, and accumulating evidence indicates that CD98hc plays a significant role in regulating integrin-mediated functions in cancer cells. Cross-linking CD98hc promotes activation of phosphatidylinositol 3-kinase (PI3K) (18) and Rap1 (19) and enhances \( \beta_1 \) integrin-mediated cell adhesion in a number of cancer cells, including breast and small cell lung cancer (14, 20), and clustering of \( \alpha_\text{IIb}\beta_\text{IIIa} \) integrin on the surface of rhabdomyosarcoma cells (21). The mechanism by which CD98hc associates with and regulates integrin function and what role this plays in transformation are unclear.

The extracellular domain of CD98hc combines with at least six different light chains to form a series of disulfide-bonded heterodimers that are involved in \( \text{L-} \) amino acid transport. The role of the light chain in CD98hc interaction with or regulation of function of \( \beta_1 \) integrins is controversial. Mutations of cysteine residues in CD98hc that disrupt covalent association with the light chain and that reduce amino acid transport also eliminate the transforming activity of CD98hc in BALB/3T3 cells (22) and cause loss of \( \beta_1 \) integrin association in low density light chain membrane fractions (21). However, these mutants still bind to free \( \beta_{1A} \) cytoplasmic tails (Tac-\( \beta_1 \)) in vitro and reverse Tac-\( \beta_1 \)-induced dominant integrin suppression in Chinese hamster ovary (CHO) cells (15). However, titration of

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** The abbreviations used are: PI3K, phosphatidylinositol 3-hydroxykinase; CHO, Chinese hamster ovary; FAK, focal adhesion kinase; mAb, monoclonal antibody; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; FACs, fluorescence-activated cell sorting; PBS, phosphate-buffered saline; PIP_2, phosphatidylinositol 3,4,5-trisphosphate; IP_3, inositol 1,4,5-tetrakisphosphate; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; ANOVA, one-way analysis of variance; FRNK, focal adhesion kinase related non-kinase.

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1 C. Feral and M. H. Ginsberg, submitted for publication.
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CD98hc by β1 tails is not the mechanism of Tac-β1 dominant suppression (15). In contrast, other evidence suggests that the cytoplasmic/transmembrane domain of CD98hc is the critical region mediating CD98hc alteration of β1 integrin surface distribution and cytoskeletal architecture in Madin-Darby canine kidney cells and reversal of Tac-β1 dominant suppression in CHO cells (15, 16). We have shown previously that cross-linking CD98hc stimulates PI3K activity in a β1 integrin-dependent manner (18). The aim of this study was to investigate the mechanism by which overexpression of CD98hc leads to cellular transformation, PI3K activation, and β1 integrins. We found that cellular transformation by overexpression of CD98hc depends on activation of PI3K mediated by focal adhesion kinase (FAK). This PI3K activation depends on the interaction of β1 integrins with CD98hc and is associated with redistribution of the integrins. Finally, we found that the CD98hc transmembrane domain is necessary and sufficient for integrin association and PI3K activation and transformation by CD98hc. This protein plays an important role in the formation of certain tumors; this study defines the CD98hc interactions and resulting signaling events that lead to transformation.

EXPERIMENTAL PROCEDURES

DNA Constructs—Human full-length CD69 was kindly provided by Dr. F. Sanchez-Madrid (Universidad Autonoma de Madrid, Madrid, Spain). The CD98hc chimeras were made by overlap PCR or restriction digestion and religation. C13T99E30 contains amino acids 1–81 of CD98hc (Swiss-Prot accession number P08195), amino acids 121–183 of CD69 (Swiss-Prot accession number Q07108), and amino acids 105–529 of CD98hc. C13T99E98 contains amino acids 1–40 of CD98hc and amino acids 82–529 of CD98hc. C13T99E59 contains amino acids 1–104 of CD98hc (Swiss-Prot accession number P08195), amino acids 121–183 of CD69 (Swiss-Prot accession number Q07108), and amino acids 105–529 of CD98hc. CD98hc(aa2–77) has a deletion of amino acids 2–77, which removes the entire cytoplasmic domain of CD98hc, maintaining the initiator methionine as well as the presumptive stop transfer sequence Val-Arg-Thr-Arg. CD98hc(aa1–61) contains amino acids 1–61 of CD98hc, maintaining the entire cytoplasmic domain of CD98hc, maintaining the initiator methionine as well as the presumptive stop transfer sequence Val-Arg-Thr-Arg.

Cell Culture and Transfection—CHO-K1 cells were obtained from the European Collection of Animal Cell Cultures and were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS), 1% nonessential amino acids, 5 μg/ml gentamycin, 50 units/ml penicillin, and 50 μg/ml streptomycin; CD25βα, and CD25β(1783F8/F795F) cells were grown in the same medium containing 10 μg/ml puromycin for selection. Transient transfection of cell lines with chimeric constructs was undertaken using Lipofectamine Plus (Invitrogen) following the manufacturer’s instructions. Under optimal conditions, a transfection efficiency of at least 60% was achieved in each cell line. Control cells were transfected with control vector pcDNA3.1. The hybridoma cell line, 4F2 (C15) was purchased from American Type Culture Collection and cultured in DMEM containing 15% FCS, 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM l-glutamine, and 1% media supplement (Sigma). Secreted antibody was purified by protein G affinity chromatography.

Construction of Stable Cell Lines—Subconfluent CHO-K1 cells were transfected with Lipofectamine plus. Cells were incubated for 30 min at 4 °C and washed twice with PBS. Samples were finally resuspended in PBS and analyzed by flow cytometry using FACS CaliburTM (BD Biosciences). Control IgG2a and IgG1 antibodies for 4F2 and CD98hc, respectively, were also used.

Clonogenic Assay—Cells (2 × 105/ml) were suspended in 0.3% (w/v) agarose in DMEM containing 1% FCS unless indicated otherwise. The mixture was overlaid over a solid layer of agarose. Cells were grown in 6-well culture dishes. Cultures were incubated in a humidified atmosphere of 5% CO2 and 95% air at 37 °C. After 6 days, colonies greater than four cells were counted under a light microscope. Cloning efficiency was calculated as a percentage of the initial number of seeded cells that formed colonies.

PI3K Activity Assay—PI3K activity was measured as described previously (21). In brief, CHO-K1 cells (5 × 105) were subjected to a standard Folch extraction, and lipid extracts containing PI3 were precipitated with an antibody at 4 °C. Immune complexes were captured using anti-protein kinase B and anti-phospho-Ser473 protein kinase B antibodies for Western blotting, the following antibodies were used: anti-protein kinase B and anti-phospho-Ser177 protein kinase B antibodies (New England Biolabs Inc., Beverly, MA), anti-FAK and anti-phospho-Tyr397 FAK antibodies (BIOSOURCE), anti-CD98 antibody (a-CD98, Santa Cruz Biotechnology), and anti-β1 integrin monoclonal antibody (mAb) (141720, Transduction Laboratories). For flow cytometry, protein A-purified 4F2 was used for identification of CD98hc and CD98hc chimeras containing the extracellular domain of CD98hc. Anti-human CD69 antibody (clone FN50, Dako Corp.) was used for identification of CD69 and chimeras containing the extracellular domain of CD69. For β1 integrin, rat clone 9EG7 was used (Pharmingen). For immunoprecipitation, anti-human β1 integrin antibody K20 (Dako Corp.) was used. Species-specific horseradish peroxidase-labeled IgG, and IgG antibodies for 4F2 and CD98hc, respectively, were also used. 32P-orthophosphate was resolved by phosphatidylserine as substrate. 3-Phosphorylated lipids were resolved by phosphatidylinositol 3,4,5-Trisphosphate (PIP3)—radioligand displacement assay for mass measurement of phosphatidylinositol 3-phosphate (PI3P). The mass of IP3 was measured by [3H]PiP3 (Amersham Biosciences) displacement from a recombinant PI3-glutathione 5-transferase-binding protein using a calibration curve obtained with unlabeled PI3 standards.

Immunoprecipitation and Western Blotting—Confluent cultures from 100-mm plates were quiesced overnight in 0.1% FCS and washed with PBS. Cells were lysed at 4 °C in lysis buffer containing 20 μM HEPES (pH 7.4), 150 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, and protease inhibitor mixture (Roche Applied Science). PI3K was immunoprecipitated from protein-equilibrated cell lysates using anti-PI3K antibody (Upstate Biotechnology) and phenyl-Sepharose 4B (Pharmacia) and assayed for activity using [γ-32P]ATP and phosphatidylinositol/phosphatidylinositolphosphatidylerine as substrate. 3-Phosphorylated lipids were resolved by thin layer chromatography, identified by autoradiography, and quantified by liquid scintillation counting.

Radioligand Displacement Assay for Mass Measurement of Phosphatidylinositol 3,4,5-Trisphosphate (PIP3)—PI3P levels were measured as described previously (21). In brief, CHO-K1 cells (5 × 105) were suspended on a standard Folch extraction, and lipid extracts containing PI3P were then subjected to alkaline hydrolysis, resulting in the release of the polar head group inositol 1,3,4,5-tetrakisphosphate (IP4). The mass of IP4 was measured by [3H]PiP3 (Amersham Biosciences) displacement from a recombinant PI3-glutathione 5-transferase-binding protein using a calibration curve obtained with unlabeled PI3 standards.

Cell Culture and Transfection—CHO-K1 cells were obtained from the European Collection of Animal Cell Cultures and were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum (FCS), 1% nonessential amino acids, 5 μg/ml gentamycin, 50 units/ml penicillin, and 50 μg/ml streptomycin. The cell lines GD25βα, null, GD25βα(1783F8/F795F), and GD25βα(1783F8/F795F) mutant cell lines were derived from GD25 cells upon stable transfection with cDNAs encoding the wild-type and mutant murine β1 integrin subunits, respectively (25). GD25βα null cells were grown in DMEM containing 10% FCS, 5 μg/ml gentamycin, 50 units/ml penicillin, and 50 μg/ml streptomycin; GD25βα and GD25βα(1783F8/F795F) cells were grown in the same medium containing 10 μg/ml puromycin for selection.

The aim of this study was to investigate the mechanism by which overexpression of CD98hc leads to cellular transformation, PI3K activation, and β1 integrins. We found that cellular transformation by overexpression of CD98hc depends on activation of PI3K mediated by focal adhesion kinase (FAK). This PI3K activation depends on the interaction of β1 integrins with CD98hc and is associated with redistribution of the integrins. Finally, we found that the CD98hc transmembrane domain is necessary and sufficient for integrin association and PI3K activation and transformation by CD98hc. This protein plays an important role in the formation of certain tumors; this study defines the CD98hc interactions and resulting signaling events that lead to transformation.
HCl (pH 7.4), 150 mM NaCl, and 0.02% (v/v) Tween 20 for 1 h at room temperature; and then incubated with primary antibody overnight at 4 °C. Species-specific horseradish peroxidase-conjugated antibodies were used for secondary labeling. Immunoreactive bands were identified by enhanced chemiluminescence (Amersham Biosciences) according to the manufacturer’s instructions.

Amino Acid Transport Assay—Cells (5 × 10⁶) were washed twice and resuspended in amino acid-free and Na⁺-free uptake solution containing 100 mM choline chloride, 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 10 mM HEPES (pH 7.5). After equilibration at 37 °C for 30 min, 2 μCi of [γ-³²P]l-[4,5-³H]leucine (82 Ci/mmol) containing 1 unlabeled l-leucine was added to each tube, and incubation was continued for an additional 30 min at 37 °C. Cells were then placed on ice; pelleted; and washed three times with 1 ml of ice-cold wash buffer containing 80 μM choline chloride, 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 10 mM HEPES (pH 7.5). The washed cells were then digested with 200 μl of 0.2% SDS in 0.2 M NaOH for 1 h. Protein-equilibrated aliquots of 100 μl were added to scintillation fluid containing 100 μl of 0.2 M HCl, and activity was counted in a scintillation counter.

Confocal Immunofluorescence—Cells were plated onto glass coverslips, fixed with 3% paraformaldehyde, and quenched in 50 mM NH₄Cl. Nonspecific binding sites were blocked using 0.2% fish skin gelatin in PBS. Cells were then incubated sequentially with (i) 4F2 or fluorescein isothiocyanate-conjugated FN50, 9EG7, anti-phospho-FAK antibody, or IgG₂ and IgG₃, negative control antibodies, and (ii) secondary Alexa Fluor antibodies. To assess the co-localization of CD98hc and β₁ integrin, incubation with 9EG7 was carried out overnight at 4 °C prior to fixation. In these experiments, incubation with 4F2 or fluorescein isothiocyanate-conjugated FN50 was performed last of all, after secondary labeling of the β₁ integrin. Confocal microscopy was performed with a Leica TCS NT confocal microscope system, and image analysis was performed using Leica TCS NT software.

Statistical Analysis—Results are presented as means ± S.E. Significance of the differences between means was assessed using one-way analysis of variance (ANOVA) or two-tailed Student’s t test. Values of p < 0.05 were considered significant. Unless stated otherwise, studies were performed on three to six independent occasions.

RESULTS

CD98hc-induced Anchorage- and Serum-independent Growth Is Dependent on the Level of CD98hc Expression and PI3K Activation—To investigate the role that CD98hc plays in cancer, we examined the effect of overexpressing human CD98hc in CHO cells on anchorage-independent growth in soft agarose, a cardinal feature of malignant transformation that closely correlates with xenograft growth in nude mice, human tumor invasiveness, and clinical aggressiveness (28, 29). Two clones stably expressing different cell-surface levels of CD98hc were selected. CHO cells stably expressing CD69 were used as controls. Like CD98hc, CD69 is a member of the type II transmembrane protein family. To exclude the possibility of clonal variation, three different stable clones were selected, and similar results were obtained. In addition, comparable results were obtained using a transient transfection system with transfection efficiencies >60%. Furthermore, chimeric expression did not affect β₁ integrin expression as judged by flow cytometry (data not shown). The colony-forming efficiency of CHO cells stably expressing CD68hc was significantly higher than that of vector- or CD69-transfected cells. In addition, the efficiency of colony formation was greatest in the clone with the highest level of CD98hc cell-surface expression (CD98hc) (Fig. 1A). Another feature of the transformed phenotype is the ability of cells to grow under serum-free conditions. Fig. 1B shows that overexpressing CD98hc supported anchorage-independent growth even under serum-free conditions. High saturation density is also regarded as an indicator of malignant transformation. In cell culture, the CD98hc clone exhibited higher saturation density compared with CD69-transfected cells after 10 days in culture, whereas the rate of growth was unaffected (Fig. 1C).

PI3K plays a key role in integrin activation and cellular activation and transformation (30). We therefore examined the effect of overexpressing CD98hc on PI3K activity in CHO cells. Expression of CD98hc significantly increased PI3K activity compared with expression of CD69 by 2–2.5-fold and increased phosphorylation of protein kinase B (Fig. 2A). The PI3K inhibitor LY294002 (31) caused a marked concentration-dependent inhibition of the colony-forming ability of CHO cells stably overexpressing CD98hc (IC₅₀ = 2.1 μM) (Fig. 2B). Thus, increased expression of wild-type CD98hc acts like an oncogene, stimulating serum- and anchorage-independent clonal growth. These effects are dependent on the level of CD98hc cell-surface expression and are blocked by inhibiting PI3K activation.

The Transmembrane Domain (Amino Acids 82–104) of CD98hc Is Necessary and Sufficient for PI3K Activation, Elevation of Intracellular PIP₃, and Colony Formation—CD98hc/CD69 chimeras (in which the extracellular, transmembrane, and cytoplasmic domains of CD98hc were exchanged with those of the type II membrane protein CD69 as shown in Fig. 3) were transfected into CHO cells to investigate the structure/function relationship of CD98hc to PI3K activation and trans-
formation. Stable CHO cell lines were generated expressing each chimera at comparable levels as judged by flow cytometry and Western blot analysis (Fig. 4). The membrane topography of CD98hc and each of the chimeras has been established previously (32); the C terminus is extracellular, and the N terminus is cytoplasmic. Chimeric expression did not affect β1 integrin expression as judged by flow cytometry (data not shown).

The effect of expressing CD98hc/CD69 chimeras on PI3K activity in CHO cells was examined both by in vitro kinase assay and by generation of the product PIP3 using a radioisotope dilution assay (27). The transmembrane domain of CD98hc was necessary and sufficient to activate PI3K and to elevate intracellular PIP3 levels (Fig. 3, A and B). In particular, the C98T69E98 chimera (extracellular and intracellular CD98hc and transmembrane CD98hc) and the truncation mutant CD98hc(2–77) (in which the cytoplasmic domain (amino acids 2–77) is deleted) were both able to stimulate PI3K activation and elevation of intracellular PIP3 levels. In contrast, the chimera C98T69E98 (in which the transmembrane domain of CD98hc is replaced with the CD69 transmembrane domain) did not enhance colony formation (12.1 ± 2.1, n = 4 in triplicate, mean ± S.E., p = not significant). Similar results were achieved using a transient transfection system with transfection efficiencies >60% and three different stable clones, eliminating the possibility of clonal selection. Therefore, the transmembrane domain of CD98hc is necessary and sufficient to stimulate PI3K activity, to elevate intracellular PIP3 levels, and to promote anchorage-independent growth.

The Transmembrane Domain (Amino Acids 82–104) of CD98hc Is Required and Sufficient for CD98hc and β1 Integrin Co-localization—Dual label confocal immunofluorescence microscopy was used to examine the physical relationship between native β1 integrins and CD98hc domains in vivo. We have shown previously that CD98hc constitutively associates with β1 integrins, regardless of activation state, using stimulating, inhibitory, and neutral anti-β1 integrin antibodies (18). As expected, CD98hc and β1 integrin were co-localized in the plasma membrane (Fig. 5A). However, no co-localization was observed between CD69 (labeled by FN50) and β1 integrins in CHO cells expressing CD69. To examine the biochemical basis for the interaction between CD98hc and β1 integrins, the following chimeras were used: C98T69E98 (which contains the transmembrane domain of CD98hc and the extracellular and cytoplasmic domains of CD69) and CD98hc(2–77) (which has the cytoplasmic domain (amino acids 2–77) deleted) labeled by mAb FN50 and mAb 4F2, respectively. When stably expressed in CHO cells, both these mutants associated with β1 integrins (Fig. 5A). Amaris software, which analyzes three-dimensional
The transmembrane domain of CD98hc (amino acids 82–104) is necessary and sufficient for PI3K activation, elevation of intracellular PIP3, and colony formation. A, the effect of chimeras on PI3K activity. Quiescent CHO-K1 cells (5 x 10⁶) stably expressing CD69, CD98hc, or CD98hc chimeras were lysed, and PI3K activity was measured in p85α immunoprecipitates by in vitro kinase assay as described under “Experimental Procedures.” Aliquots of the lysate were subjected to SDS-PAGE and Western-blotted for the p85α subunit of PI3K to ensure equal immunoprecipitation. Radiolabeled PIP3 was resolved by thin layer chromatography, visualized by autoradiography, and quantified by liquid scintillation counting. An autoradiograph showing the 3-phosphorylated reaction product PIP3 is shown for a typical experiment. Untransfected cells stimulated with 10% FCS for 10 min or with 100 nM wortmannin for 30 min were included as controls. Results are expressed as percent activity of control untransfected cells and represent the means ± S.E. of four experiments. *, significantly different from control untransfected cells (p < 0.05, ANOVA). B, the effect of chimeras on intracellular PIP3 levels. PIP3 levels were measured in 5 x 10⁶ quiesced CHO-K1 cells stably expressing CD69, CD98hc, or CD98hc chimeras. The levels of PIP3 were quantified by an isotope dilution assay as described under “Experimental Procedures.” Results are expressed as picomoles/mg of protein and represent the means ± S.E. of four independent experiments. *, significantly different from CD69-transfected cells (p < 0.05, ANOVA). C, the effect of CD98hc chimeras on clonal growth. CHO-K1 cells stably expressing CD69, CD98hc, or CD98hc chimeras were grown in 0.3% agarose over a layer of 0.5% agarose in 1% FCS culture medium as described under “Experimental Procedures.” After 8 days, colonies greater than four cells were counted by light microscopy. Results are expressed as percent cloning efficiency and are the means ± S.E. of four independent experiments. *, significantly different from CD98-transfected cells (p < 0.05, ANOVA).

As an alternative approach to confocal microscopy, the association between CD98hc/CD69 chimeras and β1 integrins was examined by co-immunoprecipitation. Stably transfected CHO cells were lysed in 1% CHAPS and immunoprecipitated with anti-β1 integrin antibody K20. Immunoprecipitates were then separated by SDS-PAGE and Western-blotted with goat anti-CD98hc antibody. CD98hc and the CD98hc(Δ2–77) mutant were analyzed for their ability to co-immunoprecipitate with β1 integrins. The reciprocal experiment was carried out in which protein-equilibrated lysates were immunoprecipitated with 5 µg of anti-CD98hc mAb 4F2 or anti-CD69 mAb FN50 and subsequently blotted with anti-β1 integrin mAb. Fig. 6B shows that, although CD69 did not immunoprecipitate with β1 integrins, CD98hc and the chimeras containing the transmembrane domain of CD98hc did co-immunoprecipitate with β1 integrins. By contrast, those chimeras containing the transmembrane domain of CD69 did not, suggesting that the transmembrane domain of CD98hc is necessary and sufficient for integrin association. Thus, as judged by co-localization in situ and physical association, the transmembrane domain of CD98hc mediates its interaction with β1 integrins. Furthermore, only those chimeras that co-localized and associated with β1 integrins stimulated P13K activity and anchorage-independent growth, suggesting that β1 integrin association is required for CD98hc-mediated transformation.

CD98hc Alters β1 Integrin Surface Distribution and Promotes Extensive Focal Adhesion Complex Formation—CD98hc can influence integrin function (14). We therefore used confocal microscopy to examine the effect of CD98hc and chimeras on...
the localization of \( \beta_1 \) integrins and focal adhesion complexes. Expression of CD98hc altered \( \beta_1 \) integrin surface distribution, inducing loss of peripheral staining for \( \beta_1 \) integrins (Fig. 7A). Furthermore, overexpression of CD98hc promoted larger and more extensive focal adhesion complexes, consistent with increased \( \beta_1 \) integrin clustering (Fig. 7B). This phenotype was reproduced by the chimera C69T98E69, which contains the transmembrane domain of CD98hc and the extracellular and cytoplasmic domains of CD69.

**CD98hc Signaling Is \( \beta_1 \) Integrin- and FAK-dependent**—The protein-tyrosine kinase FAK plays a prominent role in integrin signaling. Overexpression of CD98hc caused a marked increase in FAK phosphorylation without affecting the level of FAK expression (Fig. 8A). Overexpression of the C69T98E69 chimera (extracellular and intracellular CD69 and transmembrane CD98hc) was sufficient to induce this increase in FAK phosphorylation (Fig. 8A).

GD25 cells derived from \( \beta_1 \) null mouse embryonic endothelial cells (GD25\( \beta_1 \), null), GD25 cells stably expressing wild-type \( \beta_1 \) integrin (GD25\( \beta_1 \)), and GD25 cells stably expressing a \( \beta_1 \) integrin subunit with point mutations (GD25\( \beta_1 \( Y783F/Y795F \)), which have been shown to have a specific deficit in \( \beta_1 \) integrin-dependent FAK activation (23, 24) were used to further examine the role of \( \beta_1 \) integrins and FAK in signaling by the transmembrane domain of CD98hc. Human full-length CD98hc, CD69, and the chimera containing only the transmembrane domain of CD98hc (C69T98E69) were transiently transfected into the GD25 cell lines. A transfection efficiency of 50–60% was achieved in all cell lines.

**Chimeras**

Fig. 4. Expression of CD98hc/CD69 chimeras in CHO-K1 cells. CD98hc/CD69 chimeras were stably transfected into CHO-K1 cells. The results from FACS analysis of expression in the stable clones using either anti-CD98 antibody 4F2 or anti-CD69 antibody are shown (A). The results from Western blot analysis of expression of CD98hc/CD69 chimeras in stable clones are also shown (B). Cell lysates were probed with anti-CD98 antibody 4F2 or anti-CD69 antibody as indicated. Analysis of expression of chimeras containing the CD69 extracellular domain was performed under nonreducing conditions, as the anti-CD98 antibody recognizes only native antigen.

Fig. 5. The transmembrane domain (amino acids 82–104) of CD98hc is required and sufficient for CD98hc and \( \beta_1 \) co-localization. A, CHO-K1 cells stably expressing CD69, CD98hc, CD98hc(Δ2–77), C69T98E69, or C98T69E98 were plated onto coverslips, and the extracellular portion was labeled with mAb FN50 (CD69 and C98T69E98) or mAb 4F2 (CD98hc, C98T69E98, and CD98hc(Δ2–77)). \( \beta_1 \) integrin was labeled with 9EG7, and immunolocalization visualized by fluorescence confocal microscopy with appropriate species-specific Alexa red (\( \beta_1 \) integrin) and Alexa green (CD98hc/CD69) secondary antibodies. In the merged panels, areas of co-localization appear yellow. Representative cells are shown. Scale bars = 2.5 μm. B, the percentage co-localization of \( \beta_1 \) integrin with CD69, CD98hc, CD98hc(Δ2–77), C69T98E69, or C98T69E98 was calculated using Leica TCS NT software. Results are expressed as percent co-localization and are the means ± S.E. of five independent experiments.
the transmembrane domain of CD98hc did not stimulate PI3K activity in GD25β1 null cells or GD25β1A(Y783F/Y795F) cells (Fig. 8B). Transient overexpression of CD98hc or the chimera C69T98E69 promoted a 2.5-3-fold increase in colony growth in semisolid agarose in GD25β1A cells; however, overexpression of these constructs did not stimulate clonal growth in GD25β1 null or GD25β1A(Y783F/Y795F) cells (Fig. 8C). These results show that the transmembrane interaction of CD98hc with β1 integrins is necessary for PI3K activation and anchorage-indepen
dent growth and furthermore suggest a role for FAK phosphorylation in these events.

To further assess the dependence of PI3K activation on FAK, CHO cells stably expressing CD69, CD98hc, or CD98hc chimeras were transiently transfected with the dominant-negative FAK-related non-kinase (FRNK) (33). The FRNK construct completely abolished the increase in PI3K activity induced by stable expression of CD98hc or the chimera containing the transmembrane domain of CD98hc (Fig. 9), demonstrating that FAK phosphorylation is required for PI3K activation.

**Amino Acids 82–86 (WALLL) of CD98hc Are Required for Integrin Association, PI3K Activation, and Anchorage-independent Growth But Not Amino Acid Transport—**To further examine functional interactions of CD98hc with β1 integrins, the truncation mutant CD98hc(Δ1–86) was used. The CD98hc(Δ1–86) mutant lacks amino acids 1–86 but is well expressed in CHO cells as shown by flow cytometry and confocal microscopy (Fig. 10, A and B). Although the chimera C69T98E69 and the truncation mutant CD98hc(Δ2–77) (which lacks the putative cytoplasmic tail) were able to co-localize with β1 integrins, to stimulate PI3K activation, and to promote anchorage-independent growth in semisolid agarose medium (Figs. 3 and 10), the CD98hc(Δ1–86) mutant was unable to co-localize with β1 integrins, failed to stimulate PI3K activity when stably expressed in CHO cells, and did not support anchorage-independent growth in semisolid agarose medium (Fig. 10, B and C). However, this mutant was still able to functionally interact with the light chain and stimulated a 2-fold increase in isoleucine transport (Fig. 10C). These results suggest that amino acids 82–86 (WALLL) at the putative cytoplasmic tail/transmembrane domain interface are required for integrin association, PI3K activation, and transformation and show that these functions are independent of amino acid transport.

**DISCUSSION**

In this study, we examined the structure/function relationship between CD98hc, integrin association, intracellular signaling, amino acid transport, and transformation. We have demonstrated the following. 1) The heavy chain of CD98 promotes anchorage- and serum-independent growth when over-expressed in CHO cells. This oncogenic activity is dependent on PI3K activation and the level of CD98hc cell-surface expression. 2) Using CD98hc chimeras, we have shown that the transmembrane domain of CD98hc is necessary and sufficient for overexpression-induced phosphorylation of FAK, activation of PI3K (and increased intracellular levels of PIP3), and anchorage-independent growth. The transmembrane domain is also necessary and sufficient for integrin association. Furthermore, only those chimeras that associate with β1 integrins stimulated PI3K activity and anchorage-independent growth. 3) Overexpression of human CD98hc alters β1 integrin surface distribution and promotes extensive focal adhesion complex formation. The transmembrane domain of CD98hc is sufficient to induce these phenotypic changes. 4) The presence of β1 integrin and FAK activation are necessary for the transmembrane domain of CD98hc to activate PI3K. 5) Using truncation mutants of CD98hc, we obtained results suggesting that the ability to promote transformation and integrin activation and signaling resides in the putative cytoplasmic/transmembrane domain interface (amino acids 82–86, WALLL). This interaction between CD98hc and β1 integrins appears to be critical for the oncogenic activity of CD98hc.

CD98hc plays an important role in tumorigenesis. Deletion of CD98hc impairs the development of teratocarcinomas from embryonic stem cells,1 and overexpression of CD98hc in NIH3T3 cells leads to anchorage-independent growth and tumor development in athymic mice (7). Furthermore, cross-linking CD98hc promotes anchorage-independent growth in small cell lung cancer cells (18). The ability of cells to grow in soft agar is a feature of anchorage independence and pathogenetic of the transformed phenotype, correlating with tumorigenicity and invasiveness of human tumors (29). Full oncogenic transformation is believed to require both serum- and anchorage-independent growth (11). We have shown here that CD98hc-transfected CHO cells are capable of both anchorage- and serum-independent growth, suggesting that overexpression of CD98hc mediates oncogenic transformation by providing signals that normally emanate from both integrins and growth factor receptors. β1 integrins play a central role in cancer (13, 34). CD98hc physically associates with β1 integrins (15, 16, 17), and we have shown previously that cross-linking CD98hc results in “integrin-like” signaling (18). However, other groups have been unable to chemically cross-link CD98hc directly to β1 integrins; hence, the interaction may not be direct (21). Nevertheless, an abundance of evidence points to the functional relevance of the
CD98hc-β1 integrin complex. In vitro binding data show that the cytoplasmic and transmembrane domains of CD98hc are both necessary and sufficient for binding to free β1A cytoplasmic tails and for the reversal of dominant suppression (32). The results presented here show that only those chimeras that bind to β1 integrins promote integrin signaling (as evidenced by FAK phosphorylation, PI3K activation, and increased intracellular levels of PIP3) and transformation. Chimeras in which the putative transmembrane domain of CD98hc is replaced with that of CD69 lost the capacity to associate and co-localize with β1 integrins, to stimulate PI3K, and to promote anchorage-independent growth. Results obtained with truncation mutants of CD98hc suggest that amino acids 82–86 of the putative CD98hc transmembrane/membrane proximal region of the cytoplasmic domain are required for this activity. Although amino acids 82–86 may mediate CD98/β1 integrin interactions, it is also possible that loss of these amino acids may result in a conformational change in the truncation mutant, disrupting CD98hc/β1 integrin association. Nonetheless, results obtained with truncation mutants CD98hc(Δ2–77) and CD98hc(Δ1–86) suggest the importance of the CD98hc transmembrane/membrane proximal region of the cytoplasmic domain in regulating β1 integrin function and transformation. Other integrin-binding proteins such as cytohesin-1, Rack-1, and skelemin also bind the membrane proximal region (35) and might contribute to the CD98hc/β1A tail interactions. CD98hc may also act as a "molecular facilitator" in the plasma membrane, regulating the association of integrins in the plane of the membrane with transmembrane domains from the superfamily members CD81, CD82, CD63, and CD53, modifying the positive or negative regulatory effects of these proteins on integrin activity.

CD98hc expression is high on the surface of human tumor cells (5, 6). However, in untransformed cells, the cyclical endogenous expression of CD98hc does not result in the display of malignant phenotypes. Hara et al. (7) have previously shown that increasing levels of CD98hc in NIH3T3 cells result in increased tumorigenicity. In this study, we have demonstrated that increasing levels of CD98hc cause increased levels of CD98hc-β1 integrin complexes with subsequent FAK phosphorylation, PI3K activation, and transformation. FAK is up-regulated in a wide variety of human epithelial cancers, with expression closely correlated to invasive potential. Furthermore, recent evidence demonstrated a direct link between FAK expression and tumor development in vivo and has therefore stimulated interest in strategies to block FAK function as a therapeutic intervention in cancer (36, 37). Similarly, constitutively active PI3K can transform chick embryonic fibroblasts (38), and a mutant p85 regulatory subunit of PI3K can transform fibroblasts in vitro (39). In addition, PI3K acting through protein kinase B has been shown to promote anchorage-independent growth (26, 40). Therefore, our results suggest that a major mechanism by which CD98hc overexpression drives transformation in human cancers is via an increase in
CD98hc-β1 integrin complexes with subsequent β1 integrin-dependent FAK phosphorylation and PI3K activation. The precise role of FAK in CD98hc-mediated transformation remains to be fully elucidated. FAK null cells and cells expressing dominant-negative FAK constructs have inefficient spreading or focal adhesion formation. Furthermore, confocal microscopy experiments in our laboratory (data not shown) confirm published results (24) that, although all three GD25 cell lines spread well on fibronectin and display focal adhesion contacts and F actin, qualitative differences in F actin and focal adhesion contacts are observed. GD25β1A cells have courser and more heterogeneous focal contacts than GD25β1 null cells, whereas GD25β1A(Y783F/Y795F) cells have finer and more uniform focal contacts. Thus, although our results suggest that FAK phosphorylation plays a direct role in CD98hc/β1 integrin-mediated PI3K activation and transformation, it remains possible that blocking integrin-mediated FAK activation blocks PI3K activation and transformation indirectly as a result of qualitative differences in spreading or focal adhesion formation.

The physical interaction of CD98hc with amino acid transporter light chains has been proposed to regulate integrins (21). CD98hc also regulates γ-L- and L-type amino acid transport (41, 42). This regulation appears to be due to disulfide-bonded heterodimer formation with a variety of light chains (41, 43). In addition to covalent association, there is also a noncovalent interaction between the heavy and light chains of CD98 (44). We used CD98hc chimeras lacking the CD98hc extracellular domain to completely eliminate the possibility of interactions with the light chain. Our results show that integrin association, PI3K activation, and stimulation of anchorage-independent growth by CD98hc are functions distinct and separable from the regulation of amino acid transport. Chimeras in which the transmembrane domain of CD98hc is replaced with that of CD69 lost the capacity to associate and co-localize with CD69, whereas the introduction of different light chains has been reported to decrease integrin association (21). However, as shown here, replacement of the entire extracellular domain of CD98hc with that of CD69 resulted in a protein that was still capable of affecting integrin function but did not stimulate isoleucine transport. These results suggest that the association of CD98hc with a light chain is not required for its interaction with integrins or for the functional regulation of integrins. The C109S CD98hc mutant, which blocks disulfide linkage to the light chain, has been reported to decrease integrin association (21). However, as shown here, replacement of the entire extracellular domain of CD98hc leaves this function intact. These data suggest that the effect of the C109S mutant on these functions cannot be ascribed simply to loss of light chain inter-

FIG. 8. Signaling by the transmembrane domain (amino acids 82–104) of CD98hc is β1 integrin- and FAK-dependent. A, CHO-K1 cells stably expressing CD69, CD98hc, or Cα1Tα1Eα2 were lysed and resolved by SDS-PAGE. Blots were probed with anti-phospho-Thr<sup>577</sup> FAK polyclonal antibody (P-FAK, upper panel) or anti-FAK mAb (lower panel). Bands were visualized by horseradish peroxidase-labeled secondary antibodies as described under "Experimental Procedures." Representative blots of three independent experiments are shown. B, GD25 cells expressing mouse wild-type β1 integrin, GD25β1 null cells, or GD25β1(Y783F/Y795F) cells were transiently transfected with CD69 (open bars), CD98hc (closed bars), or Cα1Tα1Eα2 (hatched bars) and lysed, and PI3K activity was measured as described under "Experimental Procedures." Aliquots of the lysate were subjected to SDS-PAGE and blotted for the p85α subunit of PI3K to ensure equal immunoprecipitation (upper panel). PIP<sub>3</sub>, was resolved by thin layer chromatography, visualized by autoradiography, and quantified by liquid scintillation counting (middle panel). An autoradiograph showing the 3-phosphorylated reaction product is shown for a typical experiment. Results are expressed as PI3K activity (counts/min × 10<sup>5</sup>) and represent the means ± S.E. of three independent experiments.

CD98hcβ1 integrin heterodimers, when transiently transfected with vector or 5 µg of FRNK, Cells were lysed; PI3K was immunoprecipitated; and activity was measured by an in vitro kinase assay as described under “Experimental Procedures.” Aliquots of the lysate were subjected to SDS-PAGE and Western-blotted with anti-FAK mAb. The blots show expression of FRNK as a 55-kDa fragment. PIP<sub>3</sub>, was resolved by thin layer chromatography and quantified by liquid scintillation counting. Results are expressed as counts/min and represent the means ± S.E. of four independent experiments.

FIG. 9. Inhibition of PI3K activity by FRNK. Quiescent CHO-K1 cells stably expressing CD69, CD98hc, or CD98hc chimeras were transiently transfected with vector or 5 µg of FRNK. Cells were lysed; PI3K was immunoprecipitated; and activity was measured by an in vitro kinase assay as described under “Experimental Procedures.” Aliquots of the lysates were subjected to SDS-PAGE and Western-blotted with anti-FAK mAb. The blots show expression of FRNK as a 55-kDa fragment. PIP<sub>3</sub>, was resolved by thin layer chromatography and quantified by liquid scintillation counting. Results are expressed as counts/min and represent the means ± S.E. of four independent experiments.
Transformation by CD98hc and β1 Integrins

action and may result from a change in the conformation or glycosylation of CD98hc. This hypothesis is supported by the marked antigenic change in CD98hc caused by the C103S glycosylation of CD98hc. This hypothesis is supported by the effect of stable CD98hc(Δ1–86) transport (right panel). The data represent the means ± S.E. of four independent experiments. * significantly different from CD69-transfected cells (p < 0.05, ANOVA).

REFERENCES


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