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The role and regulation of hepatic stellate cell apoptosis in reversal of liver fibrosis

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Liver fibrosis and its end-stage disease cirrhosis are major world health problems arising from chronic injury of the liver by a variety of etiological factors including viruses, alcohol and drug abuse, the metabolic syndrome, autoimmune disease and hereditary disorders of metabolism. Fibrosis is a progressive pathological process in which wound-healing myofibroblasts of the liver respond to injury by promoting replacement of the normal hepatic tissue with a scar-like matrix composed of cross-linked collagen. Until recently it was believed that this process was irreversible. However emerging experimental and clinical evidence is starting to show that even cirrhosis is potentially reversible. Key to this is the discovery that reversion of fibrosis is accompanied by clearance of hepatic stellate cells (HSC) by apoptosis. Furthermore, proof-of-concept studies in rodents have demonstrated that experimental augmentation of HSC apoptosis will promote the resolution of fibrosis. Consequently there is now considerable interest in determining the molecular events that regulate HSC apoptosis and the discovery of drugs that will stimulate HSC apoptosis in a selective manner. This review will consider the regulatory role played by growth factors (*e.g.* NGF, IGF-1, TGFβ), death receptor ligands (TRAIL, FAS), components and regulators of extracellular matrix (integrins, collagen, matrix metalloproteinases and their tissue inhibitors) and signal transduction proteins and transcription factors (Rho/Rho kinase, Jun N-terminal Kinase (JNK), IkappaKinase (IKK), NF-κB). The potential for known pharmacological agents such as gliotoxin, sulfasalazine, benzodiazepine ligands, curcumin and tanshinone I to induce HSC apoptosis and therefore to be used therapeutically will be explored.

Keywords: apoptosis; hepatic stellate cells; liver fibrosis; NF-kB.

Introduction

Chronic liver disease is a major cause of mortality and morbidity around the world. Cirrhosis of the liver results from chronic pro-inflammatory injury processes that lead to progressive fibrosis of the liver as part of the body's wound healing and tissue remodeling mechanisms.[1,2] These pro-inflammatory insults range from viral infections (*e.g.* Hepatitis C) to autoimmune and toxic (*e.g.* alcoholic liver disease or the metabolic syndrome).[3] The subsequent liver cirrhosis results in 4000 deaths a year in the UK alone, with two thirds dying before their 65th birthday.[4] Understanding the molecular mechanisms that regulate the hepatic inflammatory and fibrotic process is crucial for the development of therapeutic options to tackle this very disabling and fatal condition.

Central to the whole fibrotic process is the generation and proliferation of α-smooth muscle actin (α-SMA) positive myofibroblasts from the periportal and perisinusoidal areas.[5] Although more than one potential source for these cells exist,[6,7] the best studied and understood are hepatic stellate cells (HSC).[8] HSC reside in the space of Disse in the normal liver and their main function is storage of vitamin A and other retinoids.[9] Upon liver injury HSC undergo a remarkable transformation to an activated α-SMA positive phenotype. These cells have a high proliferative index and are the major source of fibrillar collagen production within the fibrotic liver. They also potentially mediate sinusoidal blood flow via contraction and release proinflammatory and profibrogenic cytokines such as IL-6, IL-8, MCP-1 and ICAM-1.[10,11] HSC also produce tissue inhibitors of metalloproteinases (TIMP) and this shifts the balance of the extracellular matrix towards deposition of collagen and fibrosis.[12] This progressive deposition of matrix leads to structural and functional disturbance of hepatic function and eventually death. Activation of HSC can be mimicked *in vitro* by culturing freshly isolated (quiescent) HSC on plastic, a well established model for the study of this important hepatic cell type.[13]

Reversibility of fibrosis

For many years liver fibrosis and cirrhosis was considered irreversible and liver transplantation was deemed the only therapeutic option.[14] However, there has been a paradigm

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shift in the field with increasing clinical and experimental evidence for reversibility. Biopsy based studies from patients with hepatitis C treated with pegylated interferon and ribavirin have shown consistent evidence of reversibility of fibrosis in those patients that responded to therapy.[15] In fact in one study there was evidence that 3 patients with established cirrhosis seemed to revert to virtually normal histology, although definitive evidence for the reversibility of cirrhosis has not yet been obtained and this remains a contentious area.[16] There is also evidence for reversibility in other liver diseases such as autoimmune hepatitis, primary biliary cirrhosis, hepatitis B and biliary obstruction.[17–21] Furthermore, evidence for reversibility of fibrosis has also been shown in other organs such as the kidney and heart.[22,23]

In addition there is now experimental proof that liver fibrosis can be reversed. In a seminal paper originating from the Southampton group Iredale *et al*. showed that rats injured with carbon tetrachloride (an established *in vivo* model for liver fibrosis) for 4 weeks developed quite advanced fibrosis, but were able to recover to virtually normal histology after cessation of the injury.[23] More importantly they demonstrated that apoptosis of HSC was vital for this by removing the cell type responsible for both the production of the neomatrix as well as protection of this matrix through the production of TIMP. They then went on to demonstrate the same phenomenon in another model of *in vivo* injury; bile duct ligation.[25] Again, HSC apoptosis was central to this and indeed there was a 5 fold increase in HSC apoptosis as assessed using α-SMA co-staining with terminal UDP-nick end labeling (TUNEL) during the recovery phase. It also seems that in parallel with what is seen in other cell types,[26,27] HSC susceptibility to apoptosis increases with HSC activation.

These discoveries have led to increasing interest in the regulation of HSC apoptosis and its induction as a means of treating liver fibrosis.[28] A large number of studies in the last few years have looked at the susceptibility of HSC to apoptosis *in vitro* and *ex vivo*.[29] Furthermore, pharmacological therapy designed to induce HSC apoptosis *in vivo* has been shown to accelerate recovery from liver fibrosis.[30] This review will look at the current state of knowledge with respect to HSC apoptosis and its regulation.

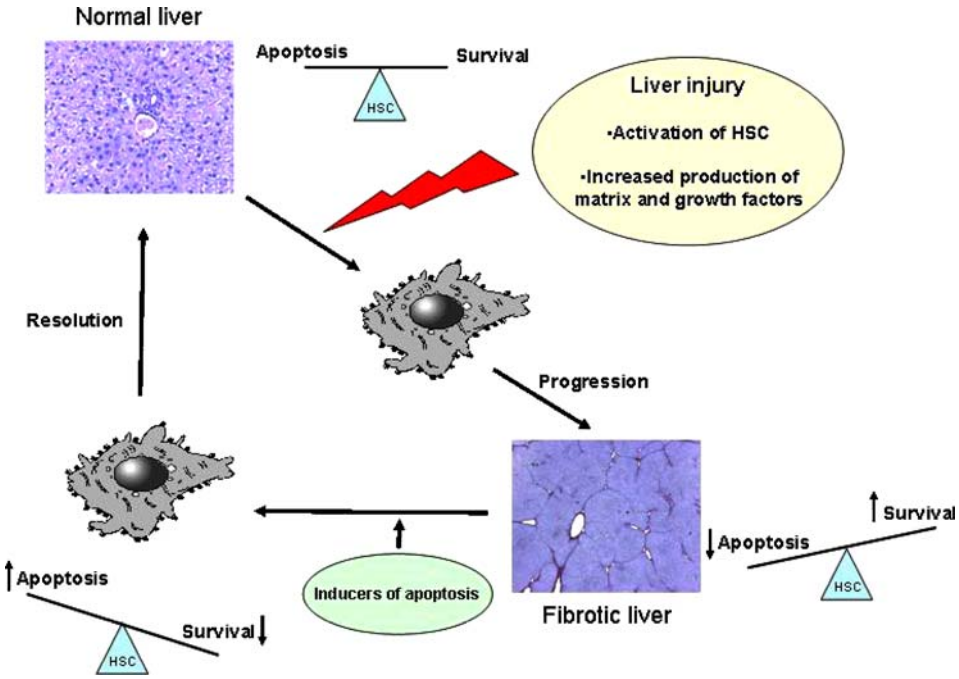
Extracellular matrix and HSC apoptosis

Extracellular matrix is increasingly being recognized as an important mediator of cell survival and proliferation.[31] Consistent with this, intact collagen-1 has been shown to promote persistence of activated HSC *in vivo*.[32] Similarly, studies in mice carrying a mutation in collagen-1 that confers resistance to the action of collagenase demonstrate that degradation of collagen-1 is critical to induction of HSC apoptosis during recovery from liver fibrosis.[33] The role of intact matrix in inhibiting HSC apoptosis was highlighted further by studies showing that lysine cross-linking of matrix by tissue transglutaminase in advanced cirrhosis appears to support HSC survival during recovery from injury and is associated with incomplete histological resolution.[34]

Integrins have also been shown to be important for HSC survival. Upon activation HSC express increasing amounts of $\alpha_v\beta_3$ integrin.[35] Inhibition of this using either a specific antibody or inhibitory RNA results in HSC apoptosis associated with a reduction in the Bcl-2/Bax ratio as well as an increase in caspase 3 activity. When activated HSC *in vitro* are treated with the pentapeptide GRGDS (Gly-Arg-Gly-Asp-Ser) that is known to antagonize integrins they undergo apoptosis.[36] This is accompanied by an increase in p53 levels as well as a reduced Bcl-2/Bax ratio. These studies suggest that integrins provide essential survival signals to activated HSC.

Consistent with this, degradation of matrix appears to promote HSC apoptosis. Indeed treatment of cells *in vitro* with recombinant matrix metalloproteinase-9 (MMP-9) stimulates HSC apoptosis.[35] In addition, treatment of HSC with an inhibitor of MMP activity promotes survival. TIMP-1 treatment of activated HSC exerts an anti-apoptotic effect that operates via its ability to inhibit MMP activity.[37] *In vivo* experiments confirmed these findings. Mice in which hepatic TIMP-1 is over-expressed by virtue of a liver-specific TIMP-1 transgene were chronically injured with CCl_4 and compared with wild type mice for their ability to undergo spontaneous resolution of fibrosis upon subsequent cessation of injury.[38] The transgenics displayed reduced ability to resolve fibrosis, did not undergo the diminution in numbers of α-SMA positive activated HSC observed in wild type animals and maintained elevated levels of procollagen I mRNA. In addition, there was a lower level of induction of active MMP-2 in the livers of recovering transgenics compared with wild type. This may be explained by the fact that apoptosis of HSC results in marked activation of pro-MMP2 (the precursor of active MMP-2).[39] The same investigators also confirmed the effect of TIMP-1 on HSC apoptosis *in vitro* and showed that the treatment of the cells resulted in reduced caspase 3 activity.[38] TIMP-1 has been shown to be an inhibitor of apoptosis in many mammalian cells by activating focal adhesion kinases, phosphatidylinositol 3-kinase, and ERKs resulting in down regulation of caspase-dependent pathways of cell death.[40] Similar pathways may operate in HSC or alternatively TIMP-1 may protect HSC from apoptosis via a mechanism involving N-cadherin which is reported to be a target for MMP activity[41]. Finally, treatment of rat hepatic stellate cell with an antibody against focal adhesion kinase (FAK) induces apoptosis.[42] This kinase is a non-receptor tyrosine kinase which has been shown to be an initiator of focal

Figure 1. Schematic diagram highlighting the role of HSC apoptosis in the genesis and resolution of liver fibrosis.



adhesion formation in adherent cells as well as having roles in cell spreading, migration and proliferation. Treatment with the antibody actually led to an increase in caspase 3 and a reduction in TIMP-1 adding further evidence to the importance of the extracellular matrix for HSC survival.

These studies suggest that with liver injury the HSC are activated and stimulated to secrete increasing amounts of collagen-1 and TIMP-1. This acts to protect the cell against apoptosis and maintain the fibrogenic environment through the action of integrins. However, this does not detract from the fact that the default pathway for HSC may be for them to undergo apoptosis.[25] With resolution of fibrosis, HSC survival signals are removed and HSC undergo apoptosis. This then removes the source of TIMP with resulting increases in MMP activity and further removal of HSC survival signals. This leads to further HSC apoptosis and eventual histological resolution (see Figure 1).

The role of other cell types in HSC apoptosis

There have been limited studies of the role of resident and non-resident hepatic cells in HSC apoptosis. The potential role of the resident liver macrophage, the Kupffer cell (KC), was highlighted recently using an activated HSC/KC co-culture system. This demonstrated that upon stimulation with lipopolysaccheride, KC were able to induce activated HSC apoptosis and that this required direct intercellular contacts.[43] The process was caspase 9 dependent as inhibition of this but not caspase 3 or 8 abrogated the response. Dexamethasone, prostaglandin E2 and tumour necrosis-related apoptosis-inducing ligand (TRAIL) receptor 2 antagonists as well as down-regulation of receptor interacting protein (RIP) were also able to inhibit LPS-induced HSC apoptosis. However, there is currently no evidence that KC-mediated HSC apoptosis occurs *in vivo*.

Apoptosis of hepatocytes is thought to play a role in initiating and maintaining HSC activation associated with liver injury.[28] Similarly, there is evidence that KC engulfment of apoptotic bodies generated from hepatocytes stimulates generation of death ligands as well as TNF-α.[44] This is thought to contribute to HSC activation and liver fibrosis.

The role of macrophages in liver fibrosis was recently studied using a transgenic mouse (CD11b-DTR) in which macrophages could be selectively depleted using a human diphtheria toxin receptor.[45] When the animals had their macrophages depleted *during* CCl_4 injury there was a reduction in the amounts of collagen III and elastin deposition. This was at least partly mediated by a reduction in the number of activated HSC seen during injury. On the other hand, depletion of macrophages during recovery from fibrosis resulted in persistence of collagen III and elastin suggesting incomplete removal of the abnormal extracellular matrix. However, there was no significant difference in the number or activity of HSC during recovery with macrophage depletion. This comment not withstanding, these data provide powerful evidence for a central role in recovery for the macrophage.

Growth factors and soluble mediators

Multiple growth factors have been shown to be mitogenic to HSC. However, recent evidence suggests that as in other cell types given certain cell conditions, some growth factors can induce apoptosis in HSC. The prime example is nerve growth factor (NGF). With HSC activation *in vitro* there is increasing expression of p75; low affinity NGF receptor.[46] This is also reflected in p75 staining of HSC in normal and diseased livers *in vivo*. Treatment of activated HSC with NGF results in inhibition of proliferation as well as induction of apoptosis. Furthermore, it has recently been shown that hepatocytes express NGF during liver injury and that at the peak of this expression there is maximal HSC apoptosis.[47] This provides evidence for possible paracrine regulation *in vivo*.

Insulin-like growth factor-1 (IGF-1) has also been studied in HSC. Treatment of activated HSC with IGF-1 resulted in apoptosis despite increased DNA synthesis.[48] This was associated with down regulation of extracellular signal regulating kinase (erk) as well as a reduction of Bcl-2/Bax ratios. In another study, however, IGF-1 was shown to ameliorate HSC apoptosis induced by both serum deprivation and cycloheximide.[25] This was associated with a reduction of caspase 3 although the intracellular signaling pathways were not analysed.

Tumour necrosis factor alpha (TNF-α) has been shown to be both an anti-apoptotic and anti-proliferative factor for HSC.[49] This may in part be due to its ability to reduce CD95L expression (see below). It has also been shown that TNF-α inhibits p53 gene expression in HSC as well as increasing expression of p21WAF-1 gene.[50] However, if protein synthesis is inhibited using cycloheximide, TNF-α has been shown to induce HSC apoptosis although the mechanism is unclear.[51]

Transforming growth factor beta (TGF-β) is known to be fibrogenic and plays key stimulatory roles in HSC activation and proliferation.[3] It is also anti-apoptotic to HSC and seems to function in much the same way as TNF-α.[49,50] However as is the case with TNF-α, inhibition of protein synthesis with cycloheximide renders HSC susceptible to TGF-β-induced apoptotis.[25] In another study TGF-β was shown to be neutral in terms of apoptosis.[51] These conflicting results may be partly accounted for by differing culture conditions as well as different concentrations of the growth factor used.

The Fas/FasL (CD95/CD95L) system has also been studied in HSC. Co-staining experiments have proved that HSC express FasL *in vivo*.[49] With HSC activation there is an increase in both Fas and FasL expression in the cells.[52] There is also a resetting of the Bcl family protein ratios. Bcl-2 as well as Bcl-$_X$L levels fall whilst Bax levels may rise or remain stable according to different investigators.[53] This is consistent with the resetting of the apoptotic threshold in activated HSC making them more susceptible to apoptosis whilst increasing their proliferation. Similar systems are believed to act in cancer cells during their transformation.

Treatment of HSC with antibodies to Fas or with FasL leads to dose dependent apoptosis as assessed by annexin staining, TUNEL positivity and nucleosomal DNA fragmentation.[53,54] The anti-apoptotic effects of TNF-α and TGF-β are thought to be partly mediated by reduced expression of FasL.[49] However, another study in activated HSC suggested that protein inhibition with cycloheximide was essential for HSC apoptosis to be induced by FasL.[55] This was associated with prolonged c-Jun kinase (JNK—see below) phosphorylation as well as membrane trafficking of Fas. Inhibition of JNK phosphorylation inhibited the membrane trafficking of Fas but did not prevent the apoptotic response. This may be due to the intracellular co-localistion of Fas and FasL observed in other cell types.[56] There has only been one study looking at the modulation of the Fas/FasL system in HSC.[57] This showed that treatment of cells with interleukin-10 (a cytokine that has been shown to reduce hepatic inflammation and fibrosis *in vivo*[58]) resulted in an increase in FasL expression associated with a reduced Bcl-2/Bax ratio.

The Fas/FasL system has been studied in other hepatic cell types, which is of relevance to HSC apoptosis due to their close proximity *in vivo*. For example, patients with alcoholic liver disease have low levels of Fas and high levels of FasL on hepatocytes.[59] It is possible that this regulates adjacent HSC apoptosis. Injection of anti-Fas antibody into mice induces lethal massive hepatocyte apoptosis.[60] Fas deficient mice that are injured using bile duct ligation have reduced hepatocyte apoptosis and develop less fibrosis.[61] These experiments suggest that the role of the Fas/FasL system within the liver is complex and further studies are needed to dissect out the components that could be harnessed for therapy development. Indeed the effects of FasL on hepatocytes may stifle the therapeutic use of this pathway.

TNF-related apoptosis-inducing ligand (TRAIL) death receptors; another branch of the death receptor family; have also been studied in HSC. With HSC activation there is an increase in TRAIL receptor 1 and 2 (TRAIL-R1/DR4 and TRAIL-R2/DR5) expression with the levels of TRAIL-R2 being 103 times higher than those of TRAIL-R1.[62] There is also an increase in TRAIL expression. At the same time there are also increases in the anti-apoptotic TRAIL-R3 and TRAIL-R4 but overall the cells become increasingly susceptible to TRAIL-mediated apoptosis with activation. Interestingly in contrast to other reports[53] there was no increase in Bax expression during activation. As hepatocytes were shown not to express TRAIL-R2 it was suggested that specific TRAIL-R2 agonists could induce HSC apoptosis without causing hepatocyte death. This idea, however, is yet to be tested *in vivo*.

Interferon-α (IFN-α) has been shown to have anti-apoptotic effects in activated HSC.[63] Treatment of cells was associated with inhibition of cell cycle progression by inducing G1 arrest and also caused a reduction in caspase 3 and caspase 8 activity. Treated cells showed no difference in the gene expression of Fas, FasL, p53, p21, p27, Bcl-2, BCL-$_X$L or Bax. However, blocking the activity of Janus kinase 2 (JAK-2) abolished the effect of IFN-α. A follow up study reported that IFN-α and interferon-γ (IFNγ) work antagonistically. Whilst IFN-α elicits an anti-apoptotic response in HSC, IFN-γ induced HSC apoptosis.[64] The chaperone protein HSP70 was found to be down regulated in response to IFN-γ treatment, which promoted an increase in caspase 8 activity. Interestingly IFN-α up-regulates HSP70 expression resulting in a decrease in caspase 8 activity via a JAK-2 dependent mechanism.

HSC in normal liver are negative for somatostatin receptors. Activation of HSC is associated with expression of all five somatostatin receptors (SSTR).[65] Using various somatostatin analogues Reynaert *et al*. were unable to show any influence on human HSC apoptosis although a SSTR1 agonist reduced the migration of these cells. In contrast, another study showed a dose dependent induction of apoptosis in rat HSC using somatostatin itself.[66] These cells only expressed SSTR1-3. There was no mechanistic data provided and it was unclear whether this is attributed to species variation or due to other factors.

The possible role of sympathetic neurotransmitters in protecting HSC from apoptosis has come to prominence recently. HSC express adrenoreceptors and catecholamine biosynthesis enzymes as well as having the ability to release norepinephrine (NE).[67] Interestingly β-hydoxylase deficient mice that are unable to produce NE only develop very mild fibrosis in response to injury. Similarly, HSC isolated from these animals grow poorly in culture. Whether inhibition of sympathetic neurotransmitters or receptors will induce HSC apoptosis or not remains to be seen. On the other hand, benzodiazepine receptors have been shown to regulate apoptosis in HSC. With HSC activation there is increased expression of peripheral benzodiazepine receptors (PBR).[68] Selective ligands of this induced dose dependent HSC apoptosis mediated through protein kinase B/akt and Bad resulting in down-regulation of Bcl-2. The investigators also demonstrated the presence of PBR on activated HSC *in vivo* following treatment with CCl_4 although detailed characterization was not carried out. Quiescent cells did not express PBR. Interestingly the pro-inflammatory cytokine interleukin-1β increased PBR expression whilst the anti-inflammatory IL-10 reduced the expression of PBR.

Leptin has also recently emerged as a regulator of HSC apoptosis and a novel profibrogenic cytokine.[69] Leptin is produced by HSC upon activation and acts as a very strong mitogen for HSC through leptin receptors; as potent as platelet derived growth factor (PDGF). Additionally leptin promotes HSC survival. Treatment with leptin increases the numbers of cells in S and G2/M phases of the cell cycle as well as increasing cyclin D1 expression. Inhibitor studies revealed the leptin response to be dependent on JAK-2 phosphorylation with downstream activation of erk, akt and PI3K. Similarly, leptin treatment protected HSC from cycloheximide and TRAIL-induced apoptosis. The fact that ob/ob mice deficient of leptin have reduced fibrosis in response to liver injury compared with their wild type littermates adds further support to the potentially important role that leptin may play in liver fibrosis and potentially the regulation of HSC apoptosis.[70]

Oxidative stress is thought to play a role in liver fibrosis, especially in response to metabolic insults such as alcohol and deposition of lipids.[71] 4-hydroxynonenal (HNE) is a product of lipid peroxidation that is known to be increased in CCl_4 injured mice. At low doses this increased HSC expression of pro-collagen-1 and TIMP-1; an effect that would be expected to be anti-apoptotic (see above).[72] At higher doses though, HNE induced HSC cell death although not through a classical caspase-dependent mechanism.

Sphingosine-1-phosphate (S1P) (a metabolite of sphingolipids which are structural components of cell membranes) provides a good example of a mediator that exerts biphasic control on HSC apoptosis. At a low concentration S1P stimulates endothelial differentiation gene (edg) receptors which signal through G proteins to stimulate erk and akt phosphorylation (the latter through PI3K).[73] These pathways are anti-apoptotic to human HSC and inhibition of one or both induced apoptosis of cells upon treatment with S1P. At higher concentrations S1P induced HSC apoptosis anyway through a receptor independent pathway that involved caspase 3 activation. The relevance of this pathway to *in vivo* liver fibrosis is not clear though.

Pharmacological agents and HSC apoptosis

Perhaps the best investigated of these is the fungal metabolite gliotoxin. This will be discussed in detail later. A myriad of other agents have been reported to induce HSC apoptosis. One of the best characterised is the polyphenol compound curcumin which is the main yellow pigment in the popular spice turmeric. Treatment of activated HSC with curcumin induces apoptosis.[74] This effect is mediated by reversing the HSC activation associated down-regulation of peroxisome proliferator-activated receptor-γ (PPAR-γ). Furthermore, treatment of primary HSC with PPAR-γ ligands inhibits collagen expression

and cellular proliferation pointing to a possible antifibrotic role for this pathway. Curcumin is a PPAR-γ ligand and also reduces production of collagen α1(I) and inhibits activity of nuclear factor kappa B (NF-κB - see below). The effect of curcumin on apoptosis can be inhibited using PPAR-γ antagonists, illustrating the important role of this nuclear receptor. With HSC activation down-regulation of this receptor mediates some of the proliferative effects of PDGF.[75] Further work has shown that signaling by curcumin through PPAR-γ results in reduced TGF-β receptor expression.[76] This results in reduced TGF-β signaling which as discussed above is anti-apoptotic. Similarly, reduced TGF-β signaling results in reduction of extracellular matrix production which further reduces HSC protection against apoptosis. To date, however, no trials have shown a beneficial effect of curcumin on hepatic fibrosis *in vivo*. The involvement of PPAR-γ activation in HSC apoptosis is interesting given recent moves to use the glitazone class of drugs (agonists of PPAR-γ) to treat patients with non-alcoholic steatohepatitis induced fibrosis.

3,4-methylenedioxymetamphetamine (ecstasy or MDMA) has also been shown to induce HSC apoptosis.[77] This was initially thought to be through activation of oxidative stress pathways. Pretreatment of cells with the antioxidant pyrrolidine dithiocarbamate (PDTC), however, abrogated the accumulation of reactive oxygen species (ROI) but did not inhibit MDMA induced apoptosis. Treatment of the cells with the pro-oxidant menadione also resulted in apoptosis although with this agent PDTC successfully blocked apoptosis. This suggests that within HSC there are ROI-dependent and ROI-independent pathways to apoptosis. Of note MDMA had to be metabolized by cytochrome P450 for it to exert its apoptotic effect. The effect of ROI has also been directly investigated in cultured activated rat HSC. Superoxide treatment induced HSC apoptosis via release of cytochrome C which lead to an increase in Bax expression and caspase 3 activity. On this basis it has been proposed that superoxide induced HSC apoptosis would limit chronic fibrotic liver injury. However no *in vivo* studies were conducted.[78]

Rapamycin is a relatively new immunosuppressant used in post-transplant treatment regimes. Although there is no direct evidence on apoptosis, *in vitro* treatment of HSC resulted in reduced proliferation in response to PDGF.[79] It also reduced fibrosis in rats undergoing CCl_4 injury *in vivo*. In these animals there was reduced expression of TGF-β1, collagen 1 and reduced tissue transglutaminase activity all of which would be predicted to establish an environment in which HSC apoptosis was facilitated (see above). The investigators did not specifically address the number of apoptotic HSC present *in vivo*.

Tetrandrine is an isoquinoline alkaloid derived from the plant *Stephania tetrandra*. It has been shown to reduce HSC activation and collagen accumulation in bile duct ligated rats. The same investigators went on to study its effects on activated HSC *in vitro*.[80] They used a Simian virus 40 transformed HSC line and demonstrated a dose dependent induction of apoptosis that was associated with caspase 3 activity. It is difficult to judge the general applicability of these findings, however, given the cell line used.

There have also been a number of studies recently looking at the effects of various components of traditional Chinese medicines (TCM) on HSC apoptosis. One such study looked at the effect of tanshinone 1, an active ingredient of *salvia miltiorrhiza*. Again rat HSC transformed by Simian virus 40 were used. Treatment of these cells with tanshinone 1 resulted in apoptosis with cytochrome c release and caspase 3 activation.[81] Other studies using TCM components showed HSC apoptosis induction by *salvia miltiorrhiza* monomer IH764-3 as well Yigan Decoction.[82,83] Similarly, caffeic acid which is found in honeybee hives was shown to induce HSC apoptosis.[84] Unfortunately many of these studies are purely descriptive with no mechanistic data and it is difficult to relate the doses used to produce these effects to the doses that might be achieved *in vivo* if they were to be used for treatment purposes. Also there is limited *in vivo* data provided in these studies.

Manipulation of HSC apoptosis *in vivo*

Many of the above pharmacological agents have only been used to stimulate HSC apoptosis *in vitro*. Since the discovery of the central role of HSC apoptosis in recovery from liver fibrosis, however, increasing numbers of investigators are attempting to manipulate the process *in vivo* in animal models of hepatic fibrosis.

The fungal metabolite gliotoxin was the first agent used to stimulate HSC apoptosis *in vivo*.[30] This was based on the observation that addition of a low concentration of gliotoxin to human and rat HSC induced apoptosis. This was associated with an increase in caspase 3 and addition of the caspase inhibitor z-VAD-FMK reduced the oligonucleosomal DNA fragmentation induced by gliotoxin. *In vivo* application showed that a single injection of gliotoxin to animals injured with CCl_4 for 7 weeks accelerated resolution of the underlying fibrosis, reduced the number of activated HSC and reduced the thickness of bridging fibrotic septae.[30] This was the first paper to provide proof of concept that inducing HSC apoptosis may accelerate recovery from liver fibrosis. Of note the only effect of gliotoxin on hepatocytes *in vivo* is to induce necrosis at high concentrations.[30] Since then another study has demonstrated that administration of gliotoxin to rats during injury with thioacetamide results in less severe liver fibrosis histologically.[85] There was also a reduction in the number of activated HSC. Disappointingly, there was no evidence that the reduced fibrosis

resulted in better liver function compared with the control animals.

Due to its obvious efficacy, the mechanism by which gliotoxin induces HSC apoptosis has been the subject of further study. Although initially chosen for its ability to inhibit NF-κB (see below) it appears that gliotoxin may not induce apoptosis through this mechanism.[30] Addition of 1.5 μM gliotoxin to HSC results in it rapid accumulation with a resultant increase in glutathione levels. This was not associated with an increase in ROS, however, and antioxidants did not show efficacy in preventing gliotoxin induced apoptosis.[86] Metabolism of gliotoxin was not required for its apoptotic effect. HSC apoptosis in response to gliotoxin is potently abrogated by thiol redox active agents such as PDTC. This is thought to be mediated via a thiol redox-dependent interaction with adenine nucleotide transporter (ANT). Indeed HSC have been shown to express ANT-1 and the motility of this transporter was altered in HSC exposed to gliotoxin.[86] ANT is implicated in mitochondrial-dependent apoptosis and the fact that gliotoxin interacts with this is consistent with changes to mitochondrial membrane permeability seen in the earlier study after two hours of gliotoxin treatment.[30]

In contrast, however, another study using human HSC has suggested that oxidative stress is a requirement for cell killing by gliotoxin.[87] Pretreatment of HSC with antioxidants such as N-acetyl cysteine (NAC) reduced gliotoxin induced cell killing at 1.5 μM as well as at 7.5 μM. This was despite the fact that generation of ROS was only evident at the higher concentrations. Gliotoxin treatment of the cells in this experiment also induced a much more rapid disruption of mitochondrial membrane permeability than previously reported. This was associated with release of cytochrome c and caspase 3 activation. Inhibition of this pathway using cyclosporine and trifluoperazine did not block gliotoxin induced apoptosis in HSC suggesting that HSC are also able to undergo programmed cell death through caspase or mitochondrial-independent pathways.[87] The conflicting findings of these studies may reflect different concentrations of gliotoxin used or different experimental conditions but further work is needed to clarify the discrepancies.

Other studies have tried to inhibit apoptosis on a more global level during liver injury. The pan-caspase inhibitor IDN-6556 was used in mice undergoing injury through bile duct ligation.[88] This showed that IDN-6556 reduced hepatocyte apoptosis and reduced markers of inflammation as well as reducing the amount of fibrosis as assessed by TGF-β and collagen 1 expression. Similarly, there was reduced activation of HSC as assessed by total liver α-SMA mRNA expression and immunohistochemistry. However, there was no attempt to quantify HSC apoptosis and the fact that there was reduced collagen-1 deposition as assessed by Sirius red staining would be expected to accelerate apoptosis of HSC due to their deprivation of survival signals (see above). The authors use the findings to argue that inhibition of hepatocyte apoptosis is antifibrogenic and that this is more important than promoting HSC apoptosis. What is not clear is how treatment with IDN-6556 would have affected animals that already had established fibrosis (much more reflective of what happens in human disease). A number of other questions also arise. Would these animals have had attenuated recovery from fibrosis? Also how do the findings relate to the fact that HSC can undergo caspase independent apoptosis?[87] Will the same also be true of other models of liver disease such as CCl_4 injury?

Another recent study looked at the role of lysosomal dependent apoptosis in liver fibrosis. Cathepsin B knockout mice were injured using bile duct ligation and compared to wild types. Of note, cathepsin B is essential for lysosomal apoptosis pathways. The knockouts demonstrated reduced hepatocyte apoptosis, lower levels of cytochrome c release and lower numbers of activated HSC. There was also less collagen deposition as assessed by sirius red staining.[89] Pharmacological inhibition of cathepsin B also produced similar results. Again, there was no specific data on HSC apoptosis in this study and it would be interesting to examine the apoptotic potential *in vitro* of HSC isolated from the knockouts.

In the future targeting of HSC apoptosis *in vivo* may involve more specific therapies than those mentioned above. With this in mind two recent studies have looked at induction of HSC apoptosis using specific molecular targeting. In the first of these two adenoviruses expressing p53 or retinoblastoma protein (rb) were used to infect activated HSC *in vitro*. This resulted in HSC apoptosis as determined morphologically as well as by increases in p21 and caspase 3.[90] The second approach utilized the thymidine kinase gene with an upstream TIMP-1 promoter sequence. This was introduced into a recombinant replication-defective adenovirus that was then used to infect HSC *in vitro*. Treatment of these cells with ganciclovir resulted in HSC apoptosis as assessed by increased caspase 3 activity and annexin V-fluorescence staining.[91] The investigators intend to use this system in a bile duct ligated mouse model in the near future. Infection rates were not quoted in either of the studies and there are still major technical obstacles to surmount before such technology could be used to deliver proteins to HSC *in vivo*, but such approaches should prove valuable in the future.

The role of NF-κB and other transcription factors in HSC apoptosis

NF-κB is a dimeric transcription factor that is central to many inflammatory and immune events.[92] It has been implicated in a diverse range of diseases including rheumatoid arthritis, atherosclerosis, Alzheimer's disease,

inflammatory bowel disease, cancer and fibrosis.[93] NF-κB is formed by the dimerisation of Rel factors. There are 5 members of the Rel family — Rel A (p65), NF-κB1 (p50), NF-κB2 (p52), c-Rel and RelB.[94] These different combinations of Rel factors form various homo- and heterodimers that bind to a common DNA binding motif (κB site) found in the upstream promoter and enhancer regions of a vast number of genes that are centrally involved in the processes of immunity, inflammation, carcinogenesis and cell cycle/apoptosis control.

Possible roles for NF-κB in HSC biology have been studied for a number of years. Constitutive NF-κB is found in activated HSC where it is involved in upregulating pro-inflammatory genes such as cytokines, chemokines and adhesion molecules.[95,96] In other cell types NF-κB is involved in protection from apoptosis.[97,98] This is partly through NF-κB directed transcriptional upregulation of the anti-apoptotic proteins TRAF-1, TRAF-2, c-IAP1 and c-IAP2.[99] At the same time NF-κB has been reported to be pro-apoptotic in some cell types.[100]

Several studies provide indirect evidence of an anti-apoptotic role for NF-κB in HSC. NGF (see above) induced apoptosis of HSC is associated with inhibition of p50/p65 DNA binding detected by electromobility shift assays as well as suppression of NF-κB transcriptional activity as determined by NF-κB reporter assays[47] This relative reduction in NF-κB binding is associated with an increase in caspase 3 activity. Similarly, curcumin induction of HSC apoptosis is also associated with inhibition of NF-κB binding as well as reduction of its trans-activating activity. This was mediated through curcumin's activation of PPAR-γ.[75] MDMA by contrast increases NF-κB binding in HSC. If this increased NF-κB binding is inhibited using either the chemical NF-κB inhibitor parthenolide or using a dominant negative form of NF-κB-inducing kinase (NIK), the proapoptotic effect of MDMA is enhanced.[78] This suggests that activation of NF-κB by MDMA limits HSC apoptosis induced by this chemical. IGF-1 induction of HSC apoptosis is also associated with down regulation of NF-κB.[48] The anti-apoptotic effects of TGF-β and TNF-α on HSC are associated with increased NF-κB activity.[50] Inhibition of this by over expression of the inhibitor of kappaB (IκB) reduces the increase in the anti-apoptotic protein Bcl-$_X$L induced by TGF-β and TNF-α treatment. Although gliotoxin is a known inhibitor of NF-κB there is controversy in the literature as to whether or not its apoptotic effect on HSC is due to this inhibition.[30,87]

Initial experiments using an adenovirus system to deliver a super repressor of NF-κB to HSC, demonstrated that NF-κB inhibition had no effect on HSC activation or proliferation.[101] It did, however, sensitise the cells to TNF-α apoptosis. It is worth noting that TNF-α is normally anti-apoptotic in the absence of NF-κB inhibition (see above). More recent studies combining *in vitro* and *in vivo* work have provided definitive evidence for a role for NF-κB in protecting HSC from apoptosis.[102] Treatment of both human and rat HSC with sulfasalazine; a known anti-inflammatory drug and potent NF-κB inhibitor; at doses of 0.5 to 2 mM induced HSC apoptosis as identified by nuclear condensation. This was associated with an increase in caspase 3 activity. Of note the individual 5-ASA and sulfapyridine moieties of sulfasalzine (which do not inhibit NF-κB) had no effect on HSC apoptosis. Furthermore, sulfasalazine inhibited the activity of NF-κB-dependent reporters transfected into HSC whilst it had not effect on NF-κB-independent reporters. NF-κB activation is mediated via a kinase complex known as inhibitor of kappa B kinases (IKK) which is a selective target for the inhibitory activity of sulfasalazine.[103] The IKK complex is comprised of several protein components of which three (IKK1, IIK2 and NEMO) have been extensively studied and functionally characterised.[100] Sulfasalazine targets the catalytic components IKK1 and IKK2 which activate NF-κB by triggering a cascade of events that results in release of the transcription factor from its inhibitor (IkB-α) as well as by stimulating phosphorylation of key functional domains in the p65 (RelA) subunit of NF-κB.[104] Sulfasalazine is at best a selective IKK inhibitor that will have the ability to inhibit other kinases which may play a regulatory role in HSC apoptosis. However, further confirmatory evidence for a role for the IKK/NF-κB pathway was provided by the demonstration that a highly cell permeable peptide inhibitor of the NEMO component of the IKK complex (which forms direct interactions with IKK1 and IKK2 that are essential for their catalytic activity) dose dependently induced HSC apoptosis. Furthermore when a version of the peptide lacking two amino acids required for binding to NEMO was introduced there was no effect on HSC apoptosis. This confirms the anti-apoptotic nature of NF-κB in HSC.

Sulfasalazine was then used *in vivo* to induce HSC apoptosis. Rats injured for 4 weeks with CCl_4 were left to recover and then after 24 hours treated with either a single shot of sulfasalazine or placebo. They were then sacrificed 24 hours later and the effects of the drug on liver fibrosis recovery were assessed. The animals receiving the drug had a significant reduction in the number of HSC compared with those receiving placebo. Furthermore, there were a greater number of TUNEL/α-SMA positive cells in the treatment group consistent with greater HSC apoptosis *in vivo*. Finally, there was also a reduction in the thickness of fibrotic bands in the animals that received sulfasalazine compared to placebo. This was accompanied by decreased amounts of pro-collagen 1, α-SMA and TIMP-1 whilst there was increased MMP-2 activity.

The mechanisms by which NF-κB inhibited apoptosis were also studied. Recent reports suggest that Gadd45β induction by NF-κB is able to block c-jun

N-terminal kinase (JNK) induced apoptosis by inhibiting JKKK2/MKK7 activation of JNK.[105] JNK is largely thought to be pro-apoptotic although in some cell systems it can be protective.[106] Activated HSC were shown to express high levels of Gadd45β and sulfasalazine treatment of the cells downregulated this. There was a coincident sulfasalzine-induced phosphorylation of JNK2 with no effect on JNK1. Pharmacological inhibition of JNK activity abrogated the apoptotic response of HSC to sulfasalazine. Furthermore, the NEMO blocking peptide also downregulated Gadd45β. Its ability to induce HSC apoptosis was also blocked by JNK inhibition.

This study suggests that NF-κB protects HSC from apoptosis and that its inhibition can be used to accelerate recovery from liver fibrosis. The mechanism by which it prevents apoptosis could involve inhibition of the JKKK/JNK/AP-1 pathway. AP-1 (activator protein 1), a transcription factor, is known to be expressed in HSC and to modulate TIMP-1 promoter activity.[107] There is increasing interest in the AP-1 pathway as a modulator of death and as a target for therapeutic manipulation.[108]

However, other JNK targets may also be important. For example, JNK is known to activate pro-apoptotic Bcl-2 proteins and its inhibition by NF-κB may reduce the intracellular activity of these proteins. In support of this preliminary data has shown that JNK is not required for JunD activity in HSC cells (D Mann—personal communication). Further work needs to be done to assess the precise JNK pathways involved in HSC survival and apoptosis as well as the exact role of AP-1 in HSC survival.

Other intracellular mediators of HSC apoptosis

The intracellular pathways involved in regulation of HSC apoptosis are relatively poorly understood. As we have seen above activation of the mitogen activated protein kinases (MAPK) ERK1/ ERK2 seems to mediate the anti-apoptotic effects of TIMP-1, leptin and S1P. On the other hand, down regulation of ERK is associated with the proapoptotic action of IGF-1. Carbenoxlone is a drug that has been used since the 1960s for the treatment of peptic ulcers. Its effects on HSC in culture were recently investigated. It inhibited activation of quiescent cells as well as DNA synthesis of activated HSC. It also reduced production of α1(I) collagen mRNA. These effects were mediated through the inhibition of erk and its upstream regulators.[109] Although no apoptosis was induced this adds weight to the importance of erk in maintaining activated HSC and protecting them from apoptosis. Little is known, however, of the role of downstream targets of ERK such as c-myc, srf and elk1 in HSC.

P38MAPK phosphorylation is associated with increased expression of α1(I) collagen mRNA in HSC and this may be anti-apoptotic in itself.[50] TGF-β is known to stimulate p38MAPK phosphorylation and is also anti-apoptotic. In contrast, however, despite the fact that TNF-α has the opposite effect on p38MAPK phosphorylation, it is also anti-apoptotic (in the absence of NF-κB inhibition) suggesting multi-level control of this pathway in HSC.[50] Consistent with a role for p38MAPK in protection of HSC from apoptosis, inhibition of Rho/Rho kinase pathway (which signals upstream of p38MAPK) was recently shown to induce HSC apoptosis with an increase in caspase 3 activity.[110] Stimulation of the pathway with lysophosphatidic acid reduced histone-associated DNA fragmentation in HSC. There was no effect in either scenarios on levels of p53, Bcl-2 or Bax levels. This preliminary experimental data is still significantly lacking in sophistication, however, as both TNF-α and Rho also act upstream of many other pathways that regulate apoptosis and careful dissection of these is needed.

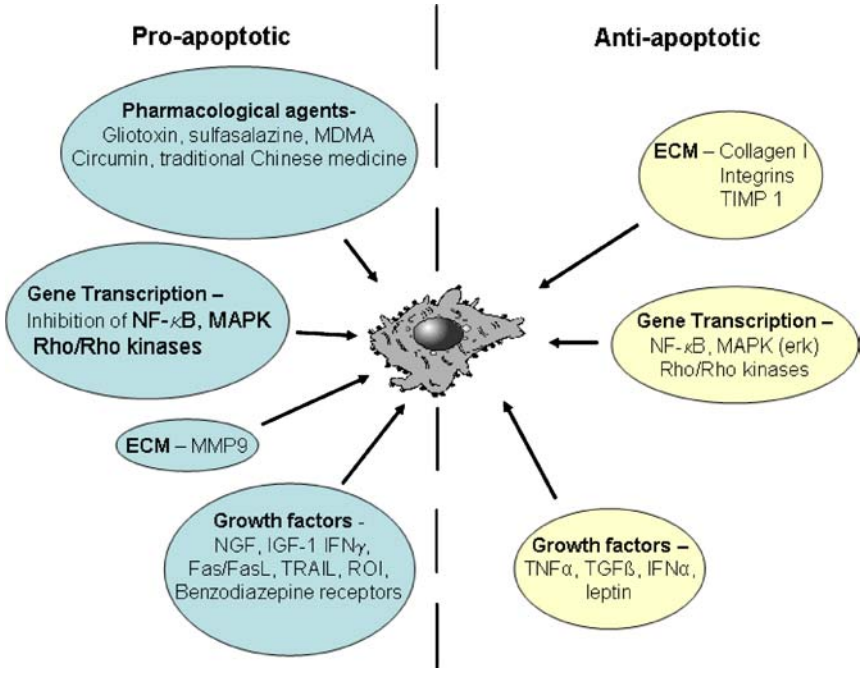
Conclusions and future research

Good progress in understanding HSC apoptosis has been made since the proof of concept experiments showing HSC apoptosis in association with reversible liver fibrosis were first published summarised in Figure 2. However, the intracellular pathways regulating HSC survival and death are only poorly understood. Despite the fact that caspase independent pathways of HSC death have been described, virtually no work has been done looking at whether or not HSC can die by non-classical apoptotic programmed cell death (PCD) pathways such as apoptosis-like PCD or necrosis-like PCD.[31] The role of mitochondrial killer proteins such as apoptosis inducing factor (AIF), endonuclease G, Smac/DIABLO and HtrA2/OMI has not been explored in HSC.[111] A better understanding of any potential role that these and other proteins play in HSC PCD will allow better and more specific targeting of therapy.

The use of the various knockouts of MEKK/MEK/MAPK pathways that are now available will also allow better functional studies of HSC apoptosis to be undertaken.[102] Similarly, the development of new specific strategies such as viral transfer of death proteins provides an exciting strategy for future treatment. The challenge will be in insuring that these proteins are only expressed in HSC; something which requires increasing characterisation of exclusive HSC proteins.[112,113]

Whilst the therapeutic possibilities highlighted by the gliotoxin and sulfasalazine studies appear promising, it is important to sound several notes of caution. Firstly, there has been no evidence produced yet showing that HSC apoptosis occurs *in vivo* in humans during treatment-initiated reversal of liver fibrosis. Secondly, there has been

Figure 2. Summary of the known regulators of HSC apoptosis.



no experimental data showing that induction of HSC apoptosis *during* liver injury can be associated with reduction in liver fibrosis and improved liver function. Thirdly, it is possible that rapid and unregulated clearance of HSC would impede resolution of fibrosis as it is these cells that produce the MMPs required for matrix clearance. Furthermore, matrix remodelling to enable maintenance of the liver's structural integrity may be compromised by rapid HSC death.

Ultimately, future studies should aim to enhance our knowledge of the kinetics of HSC clearance and how these are regulated. Promoting controlled HSC death whilst enhancing other events that promote resolution of fibrosis is needed. In addition, once fibrosis is resolved how can hepatocyte regeneration be promoted? Can stem cell therapy be deployed to enhance the liver's synthetic function? And how does all this relate to the microvasculature changes that underlie portal hypertension? These and many other questions should form the basis of future research into this ever-expanding field.

The holy grail of reversing fibrosis in the face of continuing injury still seems a long way away.

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