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Cell Death and Fibrogenesis

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Abstract

Fibrosis is a common feature of chronic liver injury and is initiated by cell death inside the liver. Hepatocyte death results in apoptotic bodies and other cellular debris, which are phagocytosed by hepatic stellate cells (HSCs), resulting in their activation, proliferation, differentiation, and matrix deposition. This profibrotic effect of cellular death is balanced by an antifibrotic effect of HSC death. Many HSC survival signals are obtained from the extracellular matrix, and active proapoptotic signals are provided by immune cells, particularly natural killer (NK) cells. Quiescent HSCs are relatively resistant to apoptotic signals but become sensitive after activation. The important role of NK cells in inducing HSC apoptosis may explain the increased fibrosis associated with immune suppression (e.g., in the transplant recipient) and HIV infection. HSCs also undergo senescence, which limits their function and sensitizes them to apoptosis.

Keywords

Hepatic stellate cells; fibrosis; cell death; apoptosis

The development of fibrosis is a common response to many types of liver injury and eventually results in cirrhosis. Liver fibrosis is, however, at one end of the spectrum of organ repair responses after injury; it is not an inevitable occurrence as demonstrated by minimal fibrosis in many patients and in many strains of mice despite chronic injury.¹ The reasons for such variability in the development of fibrosis and identification of strategies to minimize fibrosis are of great interest and lead directly to the question of what is known about the initiating and regulating events for fibrosis. In this article, we examine the role of cell death in liver fibrosis.

When considering the role of cell death in liver fibrosis, investigators have tended to focus on hepatocytes. Although this cell type is important, roles for stellate-cell and T-cell death in the development of liver fibrosis also have been identified.

TYPES OF CELLULAR DEATH

Cell death has been classified according to three main criteria: morphological appearance (apoptotic, necrotic, autophagic), enzymatic criteria (activation of caspases, nucleases, calpains, etc.), functional aspects (programmed, physiological, pathological).² In vitro experimental systems can be designed where one type of cell death occurs in a fairly uniform manner. This is in contrast to in vivo cell death that is typically due to a mixture of multiple types.

1. Apoptosis. This has a distinctive morphology with rounding up of the cell, reduction of cell volume, chromatin condensation, nuclear DNA fragmentation, plasma membrane blebbing without loss of integrity.³ Despite this very uniform set of morphological changes, at a molecular and functional level, apoptosis is heterogeneous, using a variety of different enzymes and occurring in response to different stimuli.
2. Necrosis. This is characterized by gain in cell volume, swelling of organelles, plasma membrane rupture, and loss of intracellular contents. Initially, it was thought to be an uncontrolled and essentially accidental event, but recently signal transduction pathways and catabolic enzymes have been identified.⁴
3. Autophagy. Macroautophagy is the sequestration of cytoplasmic material within double-membrane autophagosomes for bulk degradation. Eventually, the inner membrane and its contents are degraded by acidic lysosomal hydrolases. In addition to these very clear morphological features, autophagy is defined as a type of cell death occurring without chromatin condensation and is typically associated with minimal phagocytosis of the dead cell. The role of autophagy-mediated cell death in vivo is poorly understood, although a recent report provides evidence for autophagy in starvation-induced liver failure.⁵

In addition to these well-characterized modes of cell death, others have been proposed including mitotic catastrophe, which is cell death occurring after dysregulated mitosis. Excitotoxicity is a form of neuronal cell death induced by excitatory amino acids and overlaps with apoptosis and necrosis. Paraptosis is triggered by the expression of insulin-like growth factor receptor I and has some features of apoptosis but could not be prevented by caspase inhibitors. Pyroptosis is an inflammation-associated death, which was first described in macrophages infected by *Salmonella typhimurium*, and involves the activation of caspase-1. Pyronecrosis is an nucleotide binding domain and leucine rich repeat containing family, pyrin domain containing 3 (NLRP3)-dependent necrotic type cell death of macrophages triggered by *Shigella flexneri*. Unlike pyroptosis, pyronecrosis is caspase-1-independent.

CELL DEATH AS A STIMULUS FOR LIVER FIBROSIS

Hepatocyte Death

The most direct and intuitive hypothesis is that hepatocyte death provides signals for the development of liver fibrosis. The connection between hepatocyte death and fibrosis is present in chronic hepatitis C infection, where apoptosis and caspase activity correlates with the histological activity of the disease. The same has been shown in experimental models of liver fibrosis.^{6,7}

The central role of hepatic stellate cells (HSCs) in liver fibrosis makes them prime candidates for responding to apoptotic hepatocytes. This was convincingly shown by Canbay et al when they followed up on observations that phagocytosis of apoptotic bodies by macrophages results in up-regulation of transforming growth factor- β (TGF- β)⁸. HSC phagocytose apoptotic hepatocytes and up-regulate TGF- β and also collagen-1(I) mRNA. This affect is specific for apoptotic bodies and is not reproduced by fluorescent beads. Phagocytosis of apoptotic bodies also occurs in vivo, and the up-regulation of collagen-1 mRNA, but not TGF- β , requires NADPH oxidase activation.⁹ In addition to the development of a more profibrogenic phenotype, phagocytosis of apoptotic bodies makes HSCs resistant to FasL and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis via JAK/STAT and Akt/NF- κ B-dependent pathways.¹⁰ An interesting link is present with the profibrotic hormone leptin, which increases the

phagocytic ability of HSC via a Rho- and rac-1-dependent pathway.¹¹ HSC activation by phagocytosis of apoptotic cells is not restricted to hepatocytes, as phagocytosis of apoptotic disease-specific T cells has a similar effect.¹²

The above experiments provide convincing evidence that hepatocyte apoptosis directly activates HSCs. It also poses questions about the identity of signals originating from dying hepatocytes. The issue of signals from dying cells impacting on other cells is an active topic in immunology circles where cell death is known to activate dendritic cells. The signals released are termed *damage-associated molecular patterns*, and currently over 25 candidate molecules are identified.¹³ One is mammalian DNA, which is usually sequestered inside cells but can activate HSCs via the Toll-like receptor (TLR) 9. Interestingly, in addition to up-regulating TGF- β and collagen-1 mRNA, activation of TLR9 inhibited HSC chemotaxis, allowing the cells to localize to sites of hepatocyte death.¹⁴ Hepatocyte cell death also increases local adenosine concentrations, which act via the A2a receptor to induce a profibrogenic and antichemotactic phenotype.^{15–17}

Another type of cell machinery that is activated by signals from dying cells is the cytosolic protein complex termed the *inflammasome*.¹⁸ Many of its members have structural similarities with TLRs and are known to be activated by adenosine triphosphate (ATP) and uric acid. The inflammasome has recently received great attention in sensing cell death and initiating inflammatory responses and likely has an important role in sensing cell death by HSCs.¹⁹ The role of the known signals for inflammasome activation including ATP and uric acid in liver fibrosis has not been tested.

Hepatic Stellate Cell Death

Matrix deposition is a key step in the development of liver fibrosis, and HSCs are the main cell type responsible for this.¹ Based initially on morphological features and then confirmed by molecular and biochemical techniques, investigators have established that HSCs undergo differentiation from a quiescent phenotype to a myofibroblast phenotype characterized by matrix remodeling, chemotaxis, and contraction.¹ As it is clear that resolution of cirrhosis occurs in experimental and clinical models, it is important to understand the fate of differentiated HSCs, with dedifferentiation into a quiescent state or death as the main options. Analysis of HSC death in liver tissues after the induction of experimental liver fibrosis has demonstrated that HSC apoptosis occurs during the development and resolution of fibrosis and that it is associated with a decrease in HSC numbers. In contrast to the relative ease of demonstrating HSC cell death in vivo, it is difficult to induce HSC cell death in vitro.²⁰ With activation, however, HSCs become more susceptible to apoptosis. This is partly due to a decrease in levels of antiapoptotic molecules (Bcl-2 and Bcl-xL) and an increase in proapoptotic molecules (Bax).²¹ Thus analogous to other cells, activation of HSCs is associated with an increase in proliferation and death.

Proapoptotic

Many of the molecules and pathways that are known to be important in cell death have been investigated in relation to HSC apoptosis. There is an up-regulation of both CD95 and CD95-L as HSCs undergo activation. Consistent with this, anti-CD95 antibodies induce apoptosis in activated HSCs but typically require cycloheximide to maintain high levels of c-Jun-N-terminal-kinase for the anti-CD95 antibody effect to be evident.²² The antiapoptotic effects for some cytokines such as tumor necrosis factor- α (TNF- α) and TGF- β are partly due to down-regulation of CD95L on HSCs. The apparent unresponsiveness of quiescent HSCs to CD95L is because HSC do not undergo apoptosis but rather proliferation in response to CD95L. Thus, presumably in the setting of an immune response, CD95L on immune cells will stimulate expansion of the HSC pool from quiescent cells and will control

the overall size of the activated HSC pool by inducing apoptosis. Analogous to CD95, there is an up-regulation of TRAIL receptor on HSCs, with increased sensitivity to TRAIL-induced apoptosis.²³ Kupffer cells induce HSC death in cocultures with a requirement for cell contact. Significantly, this is relatively specific for activated HSCs, with 1-day-old HSCs showing almost no death.²⁴ In vivo depletion of macrophages, however, reveals a complex role for Kupffer cells, with a reduction in the deposition of matrix components if depletion occurs during liver injury and persistence of matrix components if depletion occurred during the recovery phase.²⁵ This may reflect a Kupffer cell CD95L-mediated early proliferative signal to quiescent HSCs and a later apoptotic signal to activated HSCs.

Natural killer (NK) cells have a vital role in the induction of HSC apoptosis and limiting the degree of fibrosis.²⁶ Unlike T and B cells, NK cells do not express a clonally distributed antigen receptor, but do express both inhibitory and stimulatory receptors. Many of the inhibitory receptors expressed by NK cells recognize major histocompatibility complex (MHC) class I molecules expressed by virtually all normal cells but not by some tumor cells. Some stimulatory receptors on NK cells also recognize MHC class I molecules, and others recognize a diverse range of more specialized molecules. Among the stimulatory receptors, NKG2D is the best studied. It recognizes Rae-1 and H-60 ligands in mice and MICA/B in humans. The overall activation state of NK cells is determined by integration of the signals from the inhibitory and stimulatory receptors. An important role for NK cells in liver biology is suggested by the simple observation that NK cells are abundant, making up ~10% of hepatic lymphocytes in mice and 30 to 50% in rats and humans. In general, liver NK cells express higher levels of TRAIL and perforin/granzyme and are more cytotoxic than NK cells from other organs.²⁷

NK cells are well known to limit tumor metastasis and viral infections by their cytotoxic activity.²⁸ A direct role for NK cell activation limiting liver fibrosis is demonstrated by depletion studies that showed that in the absence of NK cells, there is greater fibrosis in murine models of liver fibrosis. Conversely, activation of NK cells in mice by dsRNA polyp I:C results in less liver fibrosis. Part of the antifibrotic ability of NK cells is mediated by direct killing of HSCs and part from production of interferon gamma resulting in activation of JAK-STAT pathways.

The data from NK cell-induced cytotoxicity produce a model in which HSCs are undergoing activation, proliferation, and subsequent apoptosis. This sequence of events is termed *activation-induced cell death* (AICD) and occurs in many cell populations. AICD is not a passive event, but rather requires signals delivered by CD95-L or TRAIL. NK cells are known to have spontaneous toxicity and induce apoptosis in self cells. This is best characterized for tumor cells that have lost class I expression and have up-regulated Rae-1. These two steps remove inhibitory signals and provide stimulatory signals. In the context of liver fibrosis, the target cells are, however, nonmalignant HSCs. There are other examples in the literature of NK cells inducing apoptosis of nonmalignant cells including dendritic cells and dorsal root ganglion neurons. One important difference is that unlike many tumor cells, HSCs have not lost MHC class I, and thus may still provide inhibitory signals. However, stimulation of NKG2 is sufficient to activate NK cells even in the presence of inhibitory signals via MHC class I.²⁹ The important step in the initiation of apoptosis of HSCs appears to be up-regulation of Rae-1, and the signals responsible for this are of great interest. As activated HSCs are known to produce high levels of the immunosuppressive cytokine TGF- β , it is speculated that this factor may be down-regulated as Rae-1 is up-regulated.

The model of NK cell-induced regulation of liver fibrosis by apoptosis of HSCs provides significant insight into the relationship between the immune system and liver fibrosis. Murine studies have demonstrated that mice lacking T and B cells, and mice lacking B cells

alone, are less susceptible to liver fibrosis, and this may be in part due to expansion of NK cells. In chronic hepatitis C infection, it is clear that liver fibrosis is not associated with direct hepatocyte injury caused by the virus, which can be minimal, but does appear associated with high viral levels. The hepatitis C virus NS3/4A serine protease inhibits signaling through TLR3, which is a stimulatory signal for NK cells. This may be a form of antiviral defense designed to minimize production of antiviral cytokines, and it may also limit NK cell activation and HSC apoptosis. By similar logic, immunosuppression after organ transplantation is known to be permissive for liver fibrosis, and the use of cyclosporine along with steroids has been demonstrated to result in significant loss of cytotoxicity of NK cells. In addition, cyclosporine renders some target cells resistant to NK cell-mediated cytotoxicity. HIV represents an analogous, although more complex, scenario, but it too is associated with reduced NK-cell cytotoxicity. Alcohol consumption in experimental models limits NK-cell cytotoxicity, which contributes to greater liver fibrosis. Thus the ability of NK cells to induce HSC apoptosis and limit liver fibrosis is important to understanding how the scale of liver fibrosis is controlled during an ongoing response and also how regression of liver fibrosis occurs.

Several soluble mediators have proapoptotic effects on HSCs. After activation in vitro, and injury in vivo, there is increased expression of the low-affinity nerve growth factor (NGF) receptor p75.³⁰ NGF inhibits proliferation and induces apoptosis. Injured hepatocytes are a source for NGF and may provide paracrine regulation of HSCs.³¹ Insulin-like growth factor-1 also induces HSC apoptosis in a simple system but, unlike epidermal growth factor (EGF), increases proliferation.³² These in vitro findings, however, cannot be extrapolated to the in vivo system, because in the setting of other proapoptotic signals, insulin-like growth factor-1 reduced apoptosis.³³ TNF- α and TGF- β are both anti-apoptotic but have opposite effects on proliferation with TNF- α reducing and TGF- β stimulating proliferation.³⁴

In addition to apoptosis, another, more recently identified, mode of limiting HSC function is senescence.³⁵ This is defined as a state of permanent replicative arrest and was originally discovered in cultured primary cells. Senescence cells have a distinct morphology, gene expression, and chromatin structure.³⁵ Specific genes including P53 and Rb are required. Using senescence-specific markers, investigators have found a large number of senescent cells in the fibrotic hepatic scar.³⁶ In mice lacking the molecular pathways required for senescence, the number of senescent cells is decreased and liver fibrosis is increased. In an analogous manner, resolution of liver fibrosis is impaired in the mice lacking the molecular pathways of senescence. There appears to be a direct link between senescence and the NK cell-mediated HSC apoptosis described above, because as HSCs undergo senescence, they up-regulate immune surveillance pathways that enhance NK-cell killing of senescent cells.

Antiapoptotic

A large number of molecules have antiapoptotic properties for HSCs, several of which are part of the extracellular matrix. The evidence is best for collagen-1, which induces persistence of activated HSCs.³⁷ Although collagen may be considered a cell survival factor, it can still be an important regulator of HSC apoptosis as demonstrated by the fact that in mice with a mutant collagen resistant to collagenase, there is less HSC apoptosis.³⁷ Other inhibitors of matrix breakdown, such as cross-linking, have the same effect of decreasing HSC apoptosis.³⁸ As predicted from this, degradation of matrix promotes HSC apoptosis, and inhibiting matrix degradation promotes HSC survival.³⁹

Nonmatrix molecules with an antiapoptotic effect on HSCs include leptin, which has a very close relationship to obesity in the fed state.¹¹ Leptin has multiple effects on immunity, inflammation, and hematopoiesis in addition to its original characterization as a satiety factor. Mice lacking the leptin molecule (*ob/ob*) are resistant to the development of fibrosis.

HSC apoptosis induced by CD95-L, TNF- α , and TRAIL, but not UV irradiation, was reduced by leptin in a receptor-specific and Akt-dependent manner.^{11,40} There are also indirect effects of leptin on Kupffer cells including up-regulation of TGF- β , which directly results in HSC activation.⁴¹ Leptin also induces phagocytosis of apoptotic bodies by HSCs, making them more responsive to hepatocyte apoptosis.¹¹

The nuclear factor (NF)- κ B pathway has an important role in initiating prosurvival genes. In differentiated HSCs and myofibroblasts, there is activation of NF- κ B. At a simplistic level, up-regulation of NF- κ B signaling promotes HSC survival, and the opposite induces HSC death.⁴² Within this simple construct, there is significant complexity, because activated HSCs exhibit transcriptional repression of I κ B α , the natural inhibitor of NF- κ B. I κ B α is usually abundantly present in cells and keeps NF- κ B in an inactive state in the cytosol. Activation of an up-stream I κ B kinase (IKK) results in phosphorylation and degradation of I κ B α , releasing inhibition of NF- κ B. In HSCs, angiotensin II is important in maintaining high levels of IKK, keeping NF- κ B activated and maintaining HSC survival.⁴³ This results in a positive feedback loop as NF- κ B activation up-regulates the production of the angiotensin II precursor angiotensinogen by HSCs. The importance of this pathway has been demonstrated by the ability of drugs that block the renin-angiotensin axis at different levels to attenuate progression of experimental liver fibrosis and in humans to reduce surrogate markers of liver fibrosis.⁴⁴

Therapy

Fibrosis regresses when the injury factor is known and eliminated or neutralized. This has encouraged efforts at pharmacological interventions targeting fibrogenesis in patients with chronic liver disease.⁴⁵ Measures to decrease HSC activation, increase HSC apoptosis, increase extracellular matrix degradation, and reduce extracellular matrix deposition have been evaluated.^{46,47} Some drugs that are currently in use for other human diseases and may have antifibrosis effects are good candidates for therapeutic trials. Thiazolidinediones, and ligands for the PPAR- γ receptor are used for treatment of diabetes and may be promising in liver fibrosis. In quiescent HSCs, the PPAR- γ receptor suppresses the α 1 collagen promoter; it is down-regulated in activated HSC.⁴⁸ Oral administration of two thiazolidinediones coincident with the injury in rat models of fibrosis increased DNA binding of PPAR- γ and reduced fibrosis.⁴⁹ It remains to be seen whether or not these medications can reverse fibrosis in chronic liver injury in humans.

Pharmacological therapy designed to reduce hepatocyte apoptosis, as well as to induce HSC apoptosis (see above), may be another viable strategy. Ursodeoxycholic acid is already in use and effective in lowering serum indicators of injury in cholestatic liver disease. At least part of its effect involves inhibiting bile salt-induced hepatocyte apoptosis.⁵⁰ Caspase inhibitors, particularly the agent IDN-6556, have been effective in reducing injury or fibrosis in rodent models of liver disease including massive hepatectomy, ischemia reperfusion, and bile duct ligation. IDN-6556 has been tested in a short exposure (14 days) in patients with hepatitis C virus or other liver diseases and did reduce aspartate aminotransferase and alanine aminotransferase in patients with hepatitis C.⁵¹

In summary, the process of cell death is a vital process in the initiation, development, and resolution of liver fibrosis. Cell death inside the liver and phagocytosis of the subsequently formed apoptotic bodies by HSCs results in their activation and differentiation. The signals from dying cells are varied and result in up-regulation of matrix components as well as drawing HSCs to sites of cell death. The death of HSC is a pivotal feature in the development and resolution of liver fibrosis and is actively regulated by cells of the immune system, as well as hormones that activate the NF- κ B pathway.

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ABBREVIATIONS

AICD	activation-induced cell death
ATP	adenosine triphosphate
HSC	hepatic stellate cells
IKK	I κ B kinase
MHC	major histocompatibility complex
NF	nuclear factor
NGF	nerve growth factor
NK	natural killer
TGF-β	transforming growth factor- β
TLR	Toll-like receptors
TNF-α	tumor necrosis factor- α
TRAIL	tumor necrosis factor-related apoptosis-inducing ligand

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