Survival and apoptosis: a dysregulated balance in liver cancer

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Abstract
Background/Aims: Dysregulation of the balance between proliferation and cell death represents a protumorigenic principle in human hepatocarcinogenesis. This article aims to provide a review of the current findings about how physiological hepatocyte apoptosis is regulated and whether or not its dysregulation might contribute to the progression towards a hepatocellular carcinoma (HCC) process.

Results: Although some physiological proapoptotic molecules are downregulated or inactivated in HCC, such as Fas, p53, Bax or Bid, dysregulation of the balance between death and survival is mainly due to overactivation of antiapoptotic signals. Thus, some growth factors that mediate cell survival are upregulated in HCC, as well as the molecules involved in the machinery responsible for cleavage of their proforms to an active peptide. The expression of the pten gene is reduced or absent in almost half the HCCs and the Spred family of Ras/ERK inhibitors is also dysregulated in HCC, which consequently lead to the overactivation of relevant survival kinases: AKT and ERKs. Alterations in the expression and/or activity of molecules involved in counteracting apoptosis, such as NF-κB, Bcl-XL, Mcl-1 or c-IAP1, have also been observed in HCC. Conclusions: Therefore, therapeutic strategies to inhibit selectively antiapoptotic signals in tumour cells have the potential to provide powerful tools to treat liver cancer.

Hepatocarcinogenesis is a multistep process, in the majority of cases slowly developing with a well-defined aetiology of viral infection and chronic alcohol abuse, leading to chronic hepatitis and cirrhosis regarded as preneoplastic stages (1). A great number of factors, receptors and downstream elements of their signalling cascades regulate proliferation and apoptosis. Dysregulation of the balance between both processes represents a protumorigenic principle in human hepatocarcinogenesis, where usually there is an activation of proliferation signals and an inhibition of death process, leading to survival, and then proliferation, of affected cells. In this review, we highlight recent findings about the major molecular mechanisms known to play a physiological role in regulating hepatocyte apoptosis, and how dysregulation of these pathways might contribute to hepatocarcinogenesis.

Signalling pathways involved in physiological hepatocyte apoptosis

Apoptotic signalling within the cell is transduced mainly via two molecular pathways: the death receptor pathway (also called the extrinsic pathway) and the mitochondrial pathway (also called the intrinsic pathway) (2). Both of them activate a variety of proteases, mainly the group of proteases called caspasases (cysteinyl aspartate-specific proteases), and endonucleases, which finally degrade cellular components. Caspasases are constitutively expressed as inactive proenzymes, generally require proteolytic processing for their activation and are capable of self-activation as well as activating each other in a cascade-like process. The extrinsic and the intrinsic pathways are not mutually exclusive and some cells, hepatocytes included, require mitochondrial involvement to amplify the apoptotic signal initiated from death receptors.

Apoptotic events in hepatocytes can be regulated by different stimuli (2) that bind to death receptors in the cell membrane, such as Fas ligand (FasL), tumour necrosis factor-alpha (TNF-α) or TNF related apoptosis-inducing ligand (TRAIL), which activate the extrinsic pathway. Furthermore, other factors, particularly the transforming growth factor-beta (TGF-β), do not bind to death receptors, but its intracellular...
signals couple to the apoptotic machinery through activation of the intrinsic pathway (3).

Binding of FasL, present in natural killer cells and cytotoxic T lymphocytes, or inflammatory cytokines, such as TNF-α, to their corresponding death receptors (Fas, TNF-R1) induces the recruitment of several adapter proteins and proenzymes (procaspase – 8 and −10) at the intracellular domain of the receptor to form the so-called death-inducing signalling complex (DISC). The signal generated at DISC by activated caspases leads to cell death, which, depending on the cell type, may or may not require the involvement of mitochondria for its execution (2). TRAIL selectively induces apoptosis in various transformed cell lines but not in almost-normal tissues. It is regulated by two death receptors, TRAIL receptors 1 and 2 (TRAIL-R1 and TRAIL-R2), and two decoy receptors: TRAIL-R3 and TRAIL-R4. The expression of TRAIL-Rs has been found in most human hepatocellular carcinomas (HCCs) (4).

The intrinsic pathway is triggered by different extracellular signals that induce mitochondrial dysfunction, resulting in altered membrane permeability and mitochondrial proteins being released into the cytosol, including proapoptogenic factors such as cytochrome c, SMAC/DIABLO (second mitochondria derived activator of caspases/direct IAP binding protein with low pI), apoptosis-inducing factor (AIF) or endonuclease G, among others. The release of cytochrome c from mitochondria promotes the formation of a complex between APAF-1 and caspase-9 in a caspase-activating structure known as the apoptosome (5) (Fig. 1). Several intracellular proteins are involved in the mitochondrial-mediated regulation of apoptosis, in particular, the Bcl-2 family of proteins, which includes at least 20 members of both pro- and anti-apoptotic effects, being one of the most important regulators of the intrinsic pathway (examples of anti-apoptotic proteins are Bcl-2 and Bcl-XL; examples of proapototic members are t-Bid, Bax and Bak). These proteins exert their effects upstream of the mitochondria integrating death and survival signals. The balance between pro and antiapoptogenic members and their interactions determine the intrinsic pathway initiation.

TGF-β-induced apoptosis is associated with the removal of damaged cells and maintenance of normal cellular homeostasis and organ size (6). In the liver,
TGF-β is normally produced by stellate cells and exerts its effects by limiting the growth of hepatocytes in response to injury by inhibiting DNA synthesis, blocking cell cycle progression and inducing apoptosis. Two types of catalytic receptors (TβR-I, -II) have been described, which contain the extracellular binding domain and intracellular serine/threonine kinase domains. Proteolytically activated TGF-β binds to TβR-II, which recruits and transphosphorylates TβR-I. Then, the transcription factors SMAD2 and SMAD3 are recruited to the receptor complex and activated by phosphorylation, being released from the complex and heterodimerizing with the mediator SMAD4, followed by translocation to the nucleus. Once in the nucleus, the activated SMADs and SMAD4 regulate transcription forming complexes with other transcriptional coregulators (6) (Fig. 1). The TGF-β response in human epithelial cell lines revealed a shared cytostatic programme that include the following: (1) activation of the cyclin-dependent kinase (CDK) inhibitors CDKN2B (which encodes INK4b, also known as p15) and CDKN1A (which encodes WAF1, also known as CIP1 and p21), with consequent retinoblastoma protein (Rb) hyperphosphorylation and E2F inhibition, and 2) repression of the growth-promoting transcription factors c-Myc, ID1, ID2 and ID3. The ability of TGF-β to induce or suppress programmed cell death varies greatly depending on the cell type (7). In hepatocytes, it has been suggested that TGF-β induces the expression of the death-associated protein kinase (DAP-kinase) as an immediate SMAD-dependent early response (8). The adaptor protein DAXX has been also implicated as a mediator of TGF-β apoptotic signals because it physically associates with TβR-II, facilitating Jun amino-terminal kinase (JNK) activation (9). GADD45 β is also an immediate-early response gene for TGF-β, whose SMAD-dependent expression is responsible for delayed activation of p38 mitogen activated protein (MAP) kinase (10). The apoptosis induced by TGF-β also has been linked to an oxidative stress process, which is required for bcl-2 downregulation and mitochondria-dependent cell death (11). This process might be associated with activation of TIEG (TGF-β-inducible early-response gene) (12) and induction of a NAD(P)H oxidase-like gene, in particular nox1 (13). Furthermore, TGF-β impairs survival signals (such as the PI-3K/AKT pathway, or c-IAPs), through activation of phosphatases and/or caspase-mediated proteolysis (14, 15). These various components of the TGF-β apoptotic programme ultimately couple the signal to the main components of the cell-death machinery (Fig. 1).

**Signalling pathways involved in hepatocyte survival**

Perturbation of hepatocyte growth regulation is associated with a number of liver diseases such as fibrosis and cancer. These diseases are mediated by a network of growth factors and cytokines that regulate the induction of hepatocyte proliferation and apoptosis. Among the most important survival factors are several receptors of tyrosine kinases activated by growth factors, such as epidermal growth factor (EGF) and other family members (such as TGF-α, or heparin-binding epidermal growth factor-like growth factor: HB-EGF), fibroblast growth factors (FGFs) or hepatocyte growth factor (HGF). Activation of these receptors triggers the Ras/Raf/MEK1-2 /ERK (extracellular signal-regulated protein kinases) pathway and functional transcription factors, such AP-1, with the consequent induction of cell proliferation gene transcription (Fig. 1). A different pathway activated by these types of receptors and also implicated in cell progression is the lipid kinase phosphatidylinositol 3-kinase (PI-3K)/AKT/mTOR/p70 S6 kinase pathway (Fig. 1). In this second pathway, the phosphatase PTEN, a tumour suppressor gene product, plays an important regulatory role, as it is able to inhibit it (for a review: see (16) Breuhahn et al).

The EGF/TGF-α family represents a transmembrane-anchored molecule that can be cleaved by proteolytic shedding (e.g. by TNF-α-converting enzyme (TACE/ADAM17)). Upon ligand binding, the EGFR (four members known: Her/Erb-1, -2, -3, -4) form homo or heterodimers and initiate signal transduction through phosphorylation of intracytoplasmic tyrosine residues and recruitment of proteins with Src homology 2 domains (Grb2, Shc), which activate multiple downstream pathways such as ERK, c-JNK or p38MAPK (Fig. 1). TGF-α has been proved to be a physiological regulator of liver regeneration by means of an autocrine mechanism (17). The HGF is a potent growth factor for hepatocytes and binds to its receptor c-Met, resulting in receptor auto- and paraphosphorylation of adaptor proteins, followed by activation of cytoplasmic downstream effectors such as phospholipase C (PLCγ), signal transducers and activators of transcription (STATs), PI-3K and ERK1/2 (16). Stellate cells and myofibroblasts are induced to produce HGF by tumour cell products in HCC, and HGF in turn stimulates tumour cell invasiveness. Met coinmunoprecipitates with EGFR in protein extracts from normal hepatocytes, suggesting a new tumour-specific cross-talk for the activation of HGF/Met signalling by the TGF-α/EGFR axis (18).
Activation of ERK by growth factors phosphorylates cytoplasmic p90 ribosomal protein S6 kinase (RSK), leading to phosphorylation and inactivation of the proapoptotic protein Bad (Fig. 1). RSK further promotes cell survival by leading to the phosphorylation of CREB transcription factor (19). Ras also has a central role in survival signalling interacting with PI-3K and activating the AKT/protein kinase B (PKB) pathway. AKT provides strong antiapoptotic signals through its negative modulation of Raf, forkhead transcription factors and Bad (19). The PI-3K/AKT pathway is also important in modulating the mammalian target of rapamycin (mTOR), which acts as a central sensor for nutrient/energy availability, thereby regulating cell growth in response to the environment (19) (Fig. 1). AKT could also upregulate antiapoptotic signals, such as the antiapoptotic member of the Bcl-2 family, Mcl-1 (20). Furthermore, growth factors activate one of the major transcription factors associated with survival in the liver: the nuclear factor-κ B (NF-κB), which regulates antiapoptotic gene expression, such as c-FLIP, a specific inhibitor of caspase 8, cIAP1 and cIAP2, other caspase inhibitors and the antiapoptotic members of the Bcl-2 family Bcl-XL or Mcl-1 (Fig. 1). Activation of NF-κB in cells can also be triggered by several signals such as microbial agents (bacteria and viruses), and inflammatory cytokines [TNF-α and Interlukin (IL)-1β] (21).

It is also worth mentioning the Wnts family of glycoproteins that signal through binding to members of the frizzled (Fzd) family of receptors and activate the downstream effector dishevelled (Dvl), which prevents the phosphorylation of β-catenin by GSK3β and its subsequent degradation. Accumulation of β-catenin in the cytoplasm is followed by its translocation to the nucleus where it acts as a coactivator of transcription factors involved in antiapoptosis, angiogenesis, formation of the extracellular matrix and proliferation (22).

Imbalance of apoptosis/survival signals in HCC cells

Apoptosis represents a physiological way to eliminate the excess of cells during both liver development and regeneration (2). Indeed, insufficient apoptosis has been associated with development and progression of tumours of the liver and the biliary tree (2). HCC is the fifth most frequent neoplasm worldwide and the third leading cause of cancer death. To date, systemic chemotherapeutic treatment is ineffective against HCC (23), in part due to the apoptosis resistance observed in HCC cells. We will try to update the main molecular alterations reported for HCC that alter its apoptotic response.

Among the most common alterations observed in HCC are mutations in the p53 gene. As several chemotherapeutic agents require p53 to induce apoptosis, tumours with a disruption in the p53 pathway are generally resistant to chemotherapy. However, it was not so clear whether mutations in this gene were essential for liver tumour progression, as adenoviral delivery of p53 recombinant DNA into mice models bearing hepatocellular carcinomas did not apparently suppress tumour growth (24). DePinho and colleagues, in a recent work (25), have helped to clarify this point. They have demonstrated that the effect of p53 loss in HCC that is associated with chronic liver disease is dependent on the cellular context, in particular intact or dysfunctional telomeres, and they have hypothesized that a decreased p53 function might contribute to hepatocyte survival in the presence of telomere-induced chromosomal instability.

Disruption of the TGF-β pathway also occurs in HCC and might cause dysregulation of apoptosis (26). In favour of this hypothesis, recent studies have demonstrated that overexpression of SMAD3 reduces susceptibility to developing hepatocarcinoma, by sensitizing hepatocytes to apoptosis through downregulation of Bcl-2 (27). However, perturbations at receptor or SMAD levels do not appear to be as frequent as they are in colon or pancreatic cancer (28). Furthermore, expression of TGF-β is upregulated in a great percentage of HCC patients. Thus, other possibilities to disrupt TGF-β signalling might exist and they remain to be explored. EGF is an important survival signal for TGF-β-induced apoptosis in hepatocytes (29). PI-3K mediates the effect of EGF on TGF-β-induced death by acting upstream from the mitochondrial changes, probably counteracting TGF-β-induced oxidative stress (13). Indeed, some autocrine signals, such as EGF receptor (EGFR) ligands, might protect liver tumour cells from TGF-β-induced apoptosis. Dysregulation of growth factor signalling, including the EGF pathway, is well established in human HCCs (30). Furthermore, recent results have indicated that TGF-β might play a dual role in controlling apoptosis in foetal hepatocytes and hepatoma cells. On the one hand, it induces cell death, but on the other hand it could activate antiapoptotic signals, EGFR being required for this effect (31). The autocrine loop of EGFR activation would require a high activity of TACE/ADAM17 (31), the metalloprotease responsible for shedding of the proTNF-α, which is also necessary for shedding of the EGF family of growth factors (32). Although the possible role of an increased expression
of TACE/ADAM17 in the development of human HCC has barely been studied, a recent report indicates that the quantities of ADAM17 mRNA vary among different pathological types of HCC, but are significantly higher in poorly differentiated HCC than in well or moderately differentiated HCC (33). Overexpression of the TACE/ADAM17 might confer an advantage to HCC cells for impairing TGF-β-induced apoptosis through the transactivation of EGFR. The PI-3K/AKT pathway is also altered in HCC. The expression of the pten gene product is reduced or absent in almost half the HCCs and hepatocyte-specific abrogation pten expression in mice results in the development of HCCs (34). Genome-wide analyses of tumours in a mouse model of liver cancer and in HCC tissue have recently revealed a recurrent amplification in a region of human chromosome 11q22, delineating clAPI, the known inhibitor of apoptosis, and Yap, a transcription factor, as candidate oncogenes in the ampiclon (35). Interestingly, clAPI needs to be cleaved, and its function eliminated, during the induction process by TGF-β in hepatocytes, EGF being able to counteract this effect (14). Thus, overexpression of clAPI might also impair TGF-β-induced apoptosis in hepatocarcinoma cells.

A link between inflammation and liver cancer was suspected a few years ago. Chronic infectious with hepatitis B virus (HBV) and hepatitis C virus (HCV) are major risk factors for HCC. Studies have implicated members of the NF-κB/Rel family in both HBV- and HCV-induced neoplastic development of the liver (for a review: see (36) Arsura and Cavin). Different mechanisms have been proposed for activation of NF-κB by the hepatitis virus. Overall, inflammatory hepatitis might activate NF-κB by the concerted action of cytokines, such as TNF-α, chemokines or ILs, and viral proteins, which will likely promote cell survival of precancerous hepatocytes (36). Furthermore, a correlation between EGFR ligands and NF-κB activity has been obtained by studies in TGF-α/c-Myc mice, where an important role for NF-κB-inhibiting c-Myc-induced apoptosis was found to be essential for hepatocarcinogenesis (37). Two prosurvival NF-κB targets are the antiapoptotic member of the Bcl-2 family, Bcl-XL, and the member of the caspase inhibitors, XIAP, which are frequently overexpressed in murine and human HCCs (38, 39). Interestingly, the NF-κB / Bcl-XL /XIAP axis potently counteracts the TGF-β-induced apoptosis (40) and exerts a general cytoprotective effect on preneoplastic hepatocytes (41). Another alteration leading to defective apoptosis in virus-induced HCC is loss of response to Fas, either by downregulation of Fas expression (2), often coincident with loss of p53 expression, or by upregulation or over-activation of molecules that counteract its proapoptotic effect, including NF-κB or Bcl-XL (2, 42). Furthermore, HGF, through activation of the PI-3K/AKT pathway suppress Fas-mediated cell death in human HCC by inhibiting Fas-DISC formation, especially FADD and caspase 8 interaction (43).

Overexpression of Ras proteins is frequently observed in HCC (44). Furthermore, the Spred family of Ras/ERK inhibitors is dysregulated in HCC (45). Activated ras oncogene collaborates with the hepatitis B virus HBx protein to transform cells by suppressing HBx-mediated apoptosis (46). Thus, dysregulation of the Ras pathway might also be playing a role in balancing preneoplastic hepatocytes to survival in HBV- or HCV-mediated HCC.

It is worth noting that many of the genetic alterations observed in HCC led to an imbalance in the pro and antiapoptotic members of the Bcl-2 family. As mentioned above, bcl-xl is overexpressed in a great percentage of HCCs (38), as well as mcl-1 (47). In contrast, proapoptotic members of the family, such as bax or bel-Xs, are downregulated in HCC with dysfunction in the p53 gene (48). Furthermore, recent results have indicated that some proapoptotic genes of the BH-3-only family, such as bid, show decreased expression in HCC related to hepatitis B or C infection (49, 50).

During later stages in the development of liver tumours a loss in cell–cell contacts and acquisition of a fibroblastic-like phenotype is observed (51). This phenomenon, known as epithelial-to-mesenchymal transition (EMT), might contribute to increased migratory and metastatic capabilities of the cells. Cytokines such as TGF-β and extracellular matrix molecules are thought to contribute fundamentally to the microenvironmental interaction between stromal and malignant cells, and provide the basis for a broad repertoire of epithelial transdifferentiation. Interestingly, EMT of liver cells also results in enhanced resistance to apoptosis (52), probably due to upregulation of Snail, a repressor of E-cadherin expression that also has effects on cell homeostasis, inhibiting cell cycle and preventing cell death (53). A high percentage of human HCCs show high levels of β-catenin (1), either through stabilizing mutations of the β-catenin or overexpression of fzd, therefore favouring the intracellular accumulation of the protein (54). β-catenin expression leads to elevated EGFR levels in hepatocytes, and immunohistological analysis shows a high correlation between the expression of nuclear/cytoplasmic β-catenin and EGFR in most hepatoblastomas (16). β-catenin also participates in homotypic cell–cell interactions through its association with

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E-cadherin. Thus, β-catenin accumulation in HCC cells might contribute to impair E-cadherin expression, mediating the EMT process, migration and invasion. Interestingly, upregulation of β-catenin also contributes towards enhancement of HCC cell survival (55).

In summary, a significant number of the molecular events described to be altered in HCC progression are compromising the balance between survival and apoptotic signals in the preneoplastic hepatocytes. Some physiological proapoptotic molecules are downregulated or inactivated in HCC, but the balance between death and survival is mainly disrupted due to over-activation of antiapoptotic signals (Fig. 2). Cancer cells show stronger requirements of these intracellular pathways to survive. Therefore, therapeutic strategies to inhibit selectively antiapoptotic signals in tumour cells have the potential to provide powerful tools to treat liver cancer.

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