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## Primary hepatocytes and their cultures in liver apoptosis research

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### Abstract

Apoptosis not only plays a key role in physiological demise of defunct hepatocytes, but is also associated with a plethora of acute and chronic liver diseases as well as with hepatotoxicity. The

present paper focuses on the modelling of this mode of programmed cell death in primary hepatocyte cultures. Particular attention is paid to the activation of spontaneous apoptosis during the isolation of hepatocytes from the liver, its progressive manifestation upon the subsequent establishment of cell cultures and simultaneously to strategies to counteract this deleterious process. In addition, currently applied approaches to experimentally induce controlled apoptosis in this *in vitro* setting for mechanistic research purposes and thereby its detection using relevant biomarkers are reviewed.

## Keywords

Cell death; Apoptosis; Primary hepatocyte; *In vitro* culture; Assays; Biomarkers

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## Introduction

Like in all multicellular systems, liver homeostasis relies on the critical balance between cell growth and cell death. The latter is mainly accomplished by the programmed mode of apoptosis. In normal liver, the incidence of spontaneous apoptosis is very low, affecting only 0.1 and 0.05 % of all liver cells in mice and rats, respectively (Qiao and Farrell 1999), and particularly taking place in hepatocytes in the perivenous acinar region (Baier et al. 2006; Ni et al. 1994). However, in several acute and chronic liver diseases, including fulminant hepatic failure, alcoholic hepatitis, autoimmune hepatitis, acute and chronic viral hepatitis, non-alcoholic steatohepatitis, cholestasis, fibrosis and cirrhosis, apoptotic activity strongly increases (Guicciardi and Gores 2005; Malhi et al. 2006; Schattenberg et al. 2006; St-Pierre and Dufour 2012). Moreover, it has become clear in recent years that apoptosis predominates liver cell death induced by toxicants (Gomez-Lechon et al. 2002; Orrenius et al. 2011).

A number of protocols have been described to study hepatocellular apoptosis *in vivo*, including the direct administration of cell death-evoking toxicants to animals (Furukawa et al. 2000), the application of genetically modified subjects (Guicciardi et al. 2005) and the use of partially hepatectomised rodents (Baier et al. 2006). Such experiments not only raise serious ethical questions, but are also of rather limited scientific value. Indeed, apoptotic cells are barely detectable *in vivo*, as they are rapidly engulfed by neighbouring phagocytes. During *in vitro* experimentation, where phagocytosis does not take place, the full course of apoptosis can be monitored, whereby the late apoptotic phase is typically followed by secondary necrosis (Gomez-Lechon et al. 2002; Raffray and Cohen 1997). Cell lines are frequently applied experimental tools in *in vitro* apoptosis research, *in casu* in a hepatology context. However, these cells, such as HepG2 cells, are often derived from cancers and have typically acquired high resistance against apoptosis. Primary cells, directly isolated from healthy tissue, may offer a better alternative, as they display *in vivo*-like sensitivity to apoptosis, at least during short-term cultivation regimes (Schulze-Bergkamen et al. 2006).

In the present paper, a state-of-the-art overview of the use of primary hepatocytes and their cultures in liver apoptosis research is provided. Following a synopsis of the mechanisms that drive programmed cell death in the liver, the activation of spontaneous apoptosis during

isolation of hepatocytes from the liver as well as strategies to counteract this process in vitro is discussed in the first part. Experimental approaches to induce controlled apoptosis and biomarkers for its unequivocal detection in primary hepatocyte cultures are addressed in the second part.

## Mechanisms of hepatocyte apoptosis

Apoptosis relies on the proteolytic activity of an evolutionary conserved family of cysteinyl aspartate-specific proteinases or caspases. Yet, 2 major apoptotic pathways have been described, namely the extrinsic signalling cascade and the intrinsic pathway (Fig. 1). The latter is initiated by stimulating the release of cytochrome C from mitochondria, a process that is controlled by proapoptotic and anti-apoptotic B cell lymphoma 2 (Bcl-2) proteins. Liberated cytochrome C forms an apoptosome with apoptotic protease activating factor 1, deoxyadenosine triphosphate and procaspase 9. Following activation of caspase 9, the apoptosome enrolls and cleaves procaspase 3. In the extrinsic pathway, specific ligands, including Fas/CD95 ligand, tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) and TNF $\alpha$ -related apoptosis-inducing ligand (TRAIL), bind to their corresponding receptors at the cell plasma membrane surface, which induces the recruitment and cleavage of procaspase 8 (Decrock et al. 2009; Feldmann 1997; Jaeschke et al. 2002; Malhi and Gores 2008; Malhi et al. 2006; Orrenius et al. 2011; Raffray and Cohen 1997; Schulze-Bergkamen et al. 2006). In type I cells, sufficient amounts of caspase 8 are produced through this route and caspase 3 becomes directly activated. However, in type II cells, such as hepatocytes, only minimal quantities of active caspase 8 can be generated and the execution of the apoptotic response requires mitochondrial amplification. In this scenario, caspase 8 activates the proapoptotic Bid protein, which then translocates to the mitochondria, where it leads to the release of cytochrome C and apoptosome formation (Malhi and Gores 2008; Malhi et al. 2006; Orrenius et al. 2011; Schulze-Bergkamen et al. 2006). The overall outcome of the apoptotic pathways is the activation of caspase 3, being the main executor of apoptosis. In fact, caspase 3 cleaves a broad spectrum of cellular proteins, such as cytoskeletal proteins, which gives rise to the typical apoptotic phenotype, involving blebbing, cell shrinkage, cytoplasmic and nuclear condensation, DNA fragmentation and the formation of apoptotic bodies (Decrock et al. 2009; Feldmann 1997; Jaeschke et al. 2002; Malhi and Gores 2008; Malhi et al. 2006; Raffray and Cohen 1997; Schulze-Bergkamen et al. 2006).

## Activation of spontaneous apoptosis during hepatocyte isolation and cultivation

The 2-step collagenase perfusion technique is the most commonly used procedure to isolate hepatocytes from liver tissue of rodent and human origin (Papeleu et al. 2006; Seglen 1976). It is based upon the notion that calcium ions are critical for maintaining cellular adhesion. Thus, the freshly removed liver is perfused with a calcium-free medium, often supplemented with a calcium chelator, in a first run in order to disrupt calcium-dependent cell-cell junctions. In a second step, the liver is further perfused with a collagenase-containing buffer to eradicate cell-extracellular matrix (ECM) interactions (Alpini et al. 1994; Berry et al. 1997; Papeleu et al. 2006; Seglen 1976). The abolishment of the entire repertoire of cellular contacts triggers a proliferative response mediated by mitogen-activated protein kinase

(MAPK). The hepatocytes are hereby forced to re-enter the cell cycle from their quiescent G0 status into the G1 phase, a process evidenced by the production of proto-oncogenes, such as *c-jun* and *c-fos*. Simultaneously, an inflammatory reaction is induced, with nuclear factor-kappa beta (NF- $\kappa$ B) as master regulator, and is associated with the production of a plethora of cytokines. This is equally promoted by the inevitable occurrence of an ischaemia/reperfusion event as well as by the presence of impurities in the collagenase, such as lipopolysaccharide (LPS), used during the hepatocyte isolation procedure (Fig. 2) (Elaut et al. 2006; Paine and Andreakos 2004).

Collectively, the deleterious processes activated during hepatocyte isolation not only negatively affect the differentiated hepatocyte phenotype, but also burgeon into the onset of cell death. Indeed, expression of Bax increases already 15 min after the start of the hepatocyte isolation procedure (Kucera et al. 2006). In addition to Bax, caspase 9 translocates from the cytoplasm to the nuclei of the hepatocytes shortly after isolation (Nipic et al. 2010). When seeded in a conventional monolayer configuration on a plastic culture dish, the apoptotic response becomes progressively manifested, with high production of Bax, Bid and caspase 3 shortly after the establishment of cell cultures (Vinken et al. 2011). Thereafter, increased cleavage of caspase 3, caspase 8 and caspase 9 is observed (Bailly-Maitre et al. 2002), resulting in minimal cell viability after 4 days of cultivation (Vinken et al. 2011). Furthermore, LPS is known to induce the expression of the Fas receptor (Muschen et al. 1998) and its ligand (Yao et al. 2000) in cultured hepatocytes. It should be stressed, however, that apoptosis is not the only mode of cell death that underlies cell demise in primary hepatocyte cultures (Kucera et al. 2006; Vinken et al. 2011). In fact, 2 peaks of cell death can be discerned in this in vitro setting, both which involve apoptosis and necrosis. The cell death wave during early time points of cultivation reflects cell damage underwent during the drastic hepatocyte isolation procedure (Vinken et al. 2011). Anoikis, a particular type of apoptosis activated by cell detachment from the ECM, is likely to play an important role in this process (Elaut et al. 2006; Smets et al. 2002). When the cell culture medium is renewed on a daily basis, and thus defunct hepatocytes are removed, apoptotic and necrotic biomarkers reach minimal values around the second day of cultivation. Subsequently, a second cell death peak is noticed, which probably is a manifestation of the poor and unfavourable accommodation of the hepatocytes to the artificial cultivation conditions and thus the absence of the vital in vivo micro-environment (Vinken et al. 2011). Recently, the spontaneous cell death phenomenon taking place in primary hepatocyte cultures was found to depend on connexin43 signalling, which, similar to the in vivo situation, becomes specifically activated in hepatocellular stress conditions (Vinken et al. 2012a).

A pivotal determinant of spontaneous cell death progression in primary hepatocyte cultures is cell density (Bresgen et al. 2008; Maeda et al. 1993; Qiao and Farrell 1999; Shinzawa et al. 1995). In this respect, hepatocytes plated at low density, i.e.  $0.35 \times 10^5$  cells per  $\text{cm}^2$ , display less apoptotic activity than their counterparts seeded at high density, i.e.  $1.4 \times 10^5$  cells per  $\text{cm}^2$ , which is about half of the in vivo density, i.e.  $2\text{--}3 \times 10^5$  cells per  $\text{cm}^2$  liver tissue (Qiao and Farrell 1999). However, unlike cell survival, cell density positively correlates with the functional status of the cultured hepatocytes (Hamilton et al. 2001). Another critical parameter of cell survival in freshly isolated hepatocytes relates to the

composition of the cell culture medium. A comparison of a number of commonly used and commercially available hepatocyte culture media revealed that Williams' E medium and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer offer the lowest background of spontaneous apoptotic and necrotic cell death for freshly isolated rat hepatocytes in suspension culture (Elaut et al. 2005). More specific cell culture media, such as the University of Wisconsin solution, also are efficient in counteracting self-generated cell death in isolated hepatocytes (Fu et al. 2001).

## Strategies to counteract spontaneous apoptosis in hepatocyte cultures

### The addition of anti-apoptotic molecules to the cell culture medium

**Epidermal growth factor and hepatocyte growth factor**—Foetal bovine serum is typically added to freshly established hepatocyte cultures in order to promote cell attachment to the culture plate (Vinken et al. 2006). Foetal bovine serum enhances cell survival, yet it also negatively impacts liver-specific functionality (Tuschl et al. 2009). For this reason, and because it is a complex mixture of undefined constituents, the tendency exists to use single compounds. Among those are a set of growth factors, represented by hepatocyte growth factor (HGF) and epidermal growth factor (EGF) (Vinken et al. 2006). Growth factors are well known to protect cells from a variety of apoptotic stimuli (Ethier et al. 2003). HGF indeed significantly inhibits Fas-mediated cell death in cultures of primary human hepatocytes by reducing cleavage of caspase 8 and through induction of myeloid cell leukaemia 1 protein, an anti-apoptotic Bcl-2 protein. This is associated with the activation of the phosphatidylinositol-3 kinase (PI3K)/Akt pathway, the MAPK/extracellular signal-regulated kinase (ERK) cascade and the signal transducer and activator of transcription 3 pathway (Schulze-Bergkamen et al. 2004). HGF also upregulates the production of Bcl-2 and Bcl-xL and protects against oxidative stress (Gomez-Quiroz et al. 2008). Similarly, EGF reduces Fas-mediated apoptosis in primary mouse hepatocyte cultures by downregulation of Bid expression (Ethier et al. 2003) and through upregulation of Bcl-xL production (Musallam et al. 2001), with both processes depending on kinase activity (Ethier et al. 2003; Musallam et al. 2001). Furthermore, EGF enables the G1/S cell cycle transition in primary hepatocyte cultures, and its effect on the Fas-mediated pathway is a permissive step in this event (Gilot et al. 2005).

**Insulin and glucagon**—Insulin and glucagon control glucose levels in blood, and their actions depend on cyclic adenosine monophosphate signalling. Both hormones are frequently added to standard hepatocyte culture media, as they enhance functional hepatocellular features (Vinken et al. 2006). Insulin, together with EGF, was reported to reduce spontaneous cell death in primary hepatocyte cultures (Bresgen et al. 2008). It also decreases the number of apoptotic cells in experimentally induced cell death in primary hepatocyte cultures by increasing Bcl-xL expression (Bilodeau et al. 2004), via inhibition of *c*-jun N-terminal kinase (JNK) (Pagliassotti et al. 2007) and through activation of Akt (Bilodeau et al. 2004; Pagliassotti et al. 2007). Likewise, Fas-mediated apoptosis in suspension cultures of primary rat hepatocytes is inhibited by glucagon by involving cyclic adenosine monophosphate-dependent protein kinase (Fladmark et al. 1997).

**Dexamethasone**—Glucocorticosteroids, such as dexamethasone, are known to retard dedifferentiation in primary hepatocyte cultures by positively affecting both cell morphology and functionality. In general, glucocorticosteroids perform these actions by interfering with gene transcription (Vinken et al. 2006). As such, dexamethasone inhibits cleavage of caspase 3, caspase 8 and caspase 9 as well as the expression of Bax and cytochrome C in primary cultures of human and rat hepatocytes. In addition, it prevents the loss of the anti-apoptotic Bcl-2 and Bcl-xL proteins and negatively affects Bad translocation to mitochondria (Bailly-Maitre et al. 2001, 2002; Bresgen et al. 2008). The stabilising effect of dexamethasone on Bcl-2 and Bcl-xL depends on the pregnane X receptor (Zucchini et al. 2005).

**Dimethylsulphoxide**—The beneficial effects of dimethylsulphoxide (DMSO) on the maintenance of the differentiated status in primary hepatocyte cultures have been described by several investigators. In general, concentrations ranging from 0.5 to 2 % DMSO are added to the hepatocyte culture medium and result in the strong promotion of liver-specific functionality (Arterburn et al. 1995; Isom et al. 1985; Vinken et al. 2006). When supplementing the hepatocyte culture medium with 1 % DMSO immediately after isolation, migration of caspase 9 into the nuclei is temporarily prevented. Activities of caspase 9 and caspase 3 are, however, lower when DMSO is added 24 h after isolation (Banic et al. 2011). Inhibition of caspase 3 maturation and apoptosis execution in cultured hepatocytes by DMSO is associated with downregulation of the apoptosis signal-regulating kinase 1/JNK-p38 pathway (Gilot et al. 2002). DMSO was therefore proposed to extend the survival of primary hepatocytes by modulating the preapoptotic cell stress response (Banic et al. 2011). DMSO also prevents cellular damage in cryopreserved hepatocytes by preventing the formation of ice crystals (Fu et al. 2001).

**Phenobarbital**—Phenobarbital, a prominent cytochrome P450 inducer, has a beneficial influence on the functionality of primary hepatocytes in vitro (Vinken et al. 2006). Exposure of cultured hepatocytes to phenobarbital is known to reduce cell detachment (Holzer and Maier 1987) and to positively affect the anti-apoptotic Bcl-2 and Bcl-xL proteins, a process involving the pregnane X receptor (Zucchini et al. 2005). However, it seems that phenobarbital is more effective in reducing experimentally induced apoptosis (Bohnenberger et al. 2001; Christensen et al. 1998; Schrenk et al. 2004) rather than spontaneous cell death in primary rat hepatocyte cultures (Bohnenberger et al. 2001).

**Trichostatin A and 5-(4-dimethylaminobenzoyl)aminovaleric acid hydroxamide**—Like in other organs, critical aspects of liver homeostasis, including cell death, are associated with alterations in the chromatin structure and hence in epigenetic regulation of gene expression patterns. In this respect, induced apoptosis in primary hepatocyte cultures is accompanied by decreased levels of DNA methyltransferase 3a (Vinken et al. 2010) and opposing effects on ERK1/2 by histone deacetylases 1 and 2 (Lei et al. 2010). The latter, together with histone acetyltransferases, mediate reversible histone acetylation. Histone acetylation is typically associated with active gene expression, while the inverse holds true for histone deacetylation. Histone deacetylase inhibitors, with trichostatin A as a prototype, reduce spontaneous apoptosis in primary hepatocyte cultures as evidenced by decreased

caspase 3 activity and lowered Bid steady-state protein levels (Vanhaecke et al. 2004). At the same time, trichostatin A effectively stabilises liver-specific functionality (Henkens et al. 2007). The trichostatin A structural analogue 5-(4-dimethylaminobenzoyl)aminovaleric acid hydroxamide also delays the onset of spontaneous apoptosis in primary hepatocyte cultures (Papeleu et al. 2007).

### **The restoration of intercellular contacts**

Hepatocytes are in intimate contact with each other and also form heterotypic interactions with surrounding nonparenchymal cells within the liver, which is a prerequisite for maintaining homeostasis. Restoration of heterotypic cell interactions by cultivation of primary hepatocytes with another cell type, whether or not from hepatic origin, therefore was thought to be a way to counteract hepatocellular dedifferentiation in vitro (Vinken et al. 2006). A well-known co-culture system is the hepatocyte-rat liver epithelial cell model, in which the maintenance of the differentiated hepatocellular phenotype is kept over extended periods of time (Guguen-Guillouzo et al. 1983) and that displays less caspase 3 activity in comparison with pure hepatocyte cultures (Vanhaecke et al. 2004).

A complementary strategy to improve the differentiated status of primary cultured hepatocytes in vitro includes the boosting of homotypic hepatocyte interactions. This can be achieved by continuously rotating hepatocytes in suspension or by using cell-repelling substrates, such as porous alginate scaffolds. This results in the formation of so-called spheroids that maintain close cell–cell contacts (Vinken et al. 2006). Although these spheroids have been suggested to allow long-term cultivation of hepatocytes, considerable cell death is observed in the core of these entities. This cell death phenomenon is mainly of necrotic nature and is likely to result from the accumulation of bile acids in the centre of the spheroids (Dvir-Ginzberg et al. 2004). E-cadherin-mediated cell–cell adhesion was found to be a critical determinant for the reduction in caspase-independent cell death in hepatocyte spheroids (Luebke-Wheeler et al. 2009).

### **The re-establishment of an extracellular matrix scaffold**

In liver, hepatocytes are supported by a broad set of ECM proteins, including collagens, proteoglycans and glycosaminoglycans, which is an indispensable condition to guarantee liver functionality (Vinken et al. 2006). Detachment of hepatocytes from the ECM backbone results in anoikis. Indeed, interaction between integrins and the ECM activates focal adhesion kinase, which triggers signalling molecules such as Akt and MAPK, ultimately resulting in the suppression of cell death (Hoshiba et al. 2007). Based on this knowledge, the re-introduction of an ECM scaffold in culture was considered an evident approach to improve cell survival in primary hepatocyte cultures.

Hepatocytes easily attach to plastic culture dishes, while they fail to do so in glass culture plates, resulting in low viability and a high number of apoptotic cells after 2 h of plating (Smets et al. 2002). When seeded on a layer of collagen type I or Matrigel<sup>®</sup>, a laminin-rich extract from Engelbreth-Holm-Swarm mouse tumour, expression of Bax, Bcl-2 and Fas is lower than in conventionally cultured hepatocytes (Qiao and Farrell 1999). In these matrices, hepatocytes switch from type II to type I Fas-mediated cell death, including

activation of caspase 3 by Fas ligand independently of Bid cleavage, activation of Bax and Bad, and cytochrome C release (Walter et al. 2008). Upon addition of a second layer of ECM proteins on top of the cultured hepatocytes, the so-called sandwich culture system, a more pronounced beneficial effect on the differentiated phenotype is observed (Dunn et al. 1991, 1989; Vinken et al. 2006), which is associated with a reduction in the number of apoptotic cells (Tuschl et al. 2009; Vanhaecke et al. 2004). Godoy and colleagues showed that focal adhesion kinase is triggered in hepatocytes seeded on dried stiff collagen, leading to activation of Akt and ERK1/2. Akt causes resistance to transforming growth factor  $\beta$ 1 (TGF $\beta$ 1)-induced apoptosis by antagonising p38. By contrast, softer collagen gel does not activate focal adhesion kinase, keeping the hepatocytes in a state where they remain sensitive to TGF $\beta$ 1-induced apoptosis. In this culture system, inhibition of p38 as well as overexpression of constitutively active Akt causes apoptosis resistance (Godoy et al. 2009). Other natural ECM matrices, such as fibronectin, also positively affect cell survival in primary hepatocyte cultures, whereas agarose is not a good cultivation substratum for hepatocytes. Furthermore, a number of synthetic scaffolds have been introduced in recent years, such as poly-*N-p*-vinylbenzyl-4-*O*-beta-D-galactopyranosyl-D-gluconamide and poly-L-lysine, which are efficient in suppressing cell death, in primary hepatocyte cultures, despite the absence of Akt signalling (Hoshiba et al. 2007).

## Strategies to experimentally induce controlled apoptosis in hepatocyte cultures

### Chemical induction of hepatocyte apoptosis

**Fas-induced apoptosis**—A frequently applied method to trigger apoptosis in primary hepatocyte cultures includes the use of monoclonal antibodies directed against the Fas receptor. However, unlike the Fas ligand, binding of the antibody to the Fas receptor does not result in the onset of a proapoptotic response per se (Fadeel et al. 1997; Legembre et al. 2003; Thilenius et al. 1997). In addition, Fas-mediated apoptosis induced by antibodies becomes typically manifested to a lesser extent compared to the *in vivo* situation (Nagata 1999; Ni et al. 1994). For this reason, Fas antibodies are frequently combined with inhibitors of gene expression or protein production, such as actinomycin D and cycloheximide, respectively (Nagata 1999; Ni et al. 1994; Rouquet et al. 1996). A more rationalised strategy that resembles the natural Fas pathway is the use of Fas ligand as such (Fu et al. 2004; Reinehr et al. 2002; Vinken et al. 2009). Fas ligand can also be presented to hepatocytes by cultivation partners. In this regard, a co-culture system consisting of primary mouse hepatocytes and 3T3 fibroblasts stably transfected with Fas ligand was found to be an effective *in vitro* model to study hepatocellular cell death, since the entire hepatocyte population undergoes apoptosis 24 h after its establishment (Schlosser et al. 2000).

**Tumour necrosis factor  $\alpha$ -induced apoptosis**—In contrast to the Fas-mediated cascade, TNF $\alpha$  signalling has pleiotropic effects on hepatocytes, including induction of apoptosis, inflammatory responses and mitogenic activity (Cosgrove et al. 2008). Like for Fas antibodies and Fas ligand, the outcome of TNF $\alpha$  in primary hepatocyte cultures is often potentiated by simultaneous exposure to transcription inhibitors, such as D-galactosamine and  $\alpha$ -amanitin (Hentze et al. 2004; Schlatter et al. 2011; Schmich et al. 2011). Cell death



evoked by TNF $\alpha$  and D-galactosamine becomes even more exacerbated when hepatocytes are co-cultured with Kupffer cells (Abou-Elella et al. 2002). The cell killing effects of TNF $\alpha$  in primary hepatocyte cultures can also be reinforced by interleukins (Boer et al. 2003), LPS (Kudo et al. 2009), oxidative stress (Han et al. 2006) and hepatotoxic drugs (Cosgrove et al. 2009). TNF $\alpha$ -mediated apoptosis in cultured hepatocytes (Schwabe et al. 2004) relies on the JNK pathway.

#### **Tumour necrosis factor $\alpha$ -related apoptosis-inducing ligand-mediated**

**apoptosis**—Of all cell death receptor ligands and corresponding signalling cascades, TRAIL has yet gained least attention in the context of in vitro modelling of hepatic apoptosis. This could be explained by the fact that TRAIL preferentially induces cell death in malignant cells and not in their normal counterparts (Corazza et al. 2009; Meurette et al. 2006). However, a number of reports described the occurrence of apoptosis in primary hepatocyte cultures upon TRAIL activation, a process boosted by free fatty acids (Malhi et al. 2007), glutathione depletion (Meurette et al. 2005) and chemotherapeutic drugs (Meurette et al. 2006).

**Transforming growth factor  $\beta$ 1-induced apoptosis**—TGF $\beta$ 1 is a multifunctional cytokine that is a potent inhibitor of hepatocellular cell growth both in vivo and in vitro (Michalopoulos and DeFrances 2005; Nguyen et al. 2007). It also induces apoptosis in primary mouse hepatocyte cultures (Lei et al. 2010; Oberhammer et al. 1996; Oberhammer et al. 1992), which depends on the involvement of reactive oxygen intermediates (Sanchez et al. 1996), the activation of p38 signalling (Yoo et al. 2003) and the upregulation of nicotinamide adenine dinucleotide phosphate oxidase Nox4 (Carmona-Cuenca et al. 2008). TGF $\beta$ 1 equally evokes cell death in cultures of periportal and perivenous rat hepatocytes and is associated with a decrease in intracellular pH and lowered sodium/hydrogen exchanger activity (Benedetti et al. 1995). Activin, another member of the TGF $\beta$  family, also causes hepatocellular apoptosis in vivo and in vitro (Hully et al. 1994; Schwall et al. 1993).

**Bile acid-induced apoptosis**—Although bile salts are naturally secreted by hepatocytes, they inherently act as toxicants due to their detergent-like character. Hepatocellular accumulation of bile salts, as occurring during cholestasis, results in the onset of apoptosis (Hirschfield et al. 2010; Perez and Briz 2009). Specifically, hydrophobic bile salts, including tauro lithocholic acid and glycochenodeoxycholic acid, induce apoptosis at low concentrations, while they cause necrosis at high concentrations. These cell death responses can be reduced by hydrophilic bile salts, such as ursodeoxycholic acid and tauro ursodeoxycholic acid (Monte et al. 2009; Patel et al. 1994; Perez and Briz 2009). In primary hepatocyte cultures, the proapoptotic actions of hydrophobic bile salts involve NF- $\kappa$ B (Wang et al. 2010), PI3K (Hohenester et al. 2010), nitric oxide (NO) (Wang et al. 2011), insulin-like growth factor 1 (Drudi Metalli et al. 2007) and protein kinase C (Wang et al. 2005). Moreover, their apoptotic outcome can be intensified in this in vitro setting by the presence of free fatty acids, such as palmitic acid (Pusl et al. 2008).

### Microbiological induction of hepatocyte apoptosis

The role of hepatocellular apoptosis in hepatitis virus infection is controversial. Thus, hepatitis B virus (HBV) was reported to induce cell death in cultures of primary human hepatocytes (Arzberger et al. 2010), although this could not be reproduced by others (Schulze-Bergkamen et al. 2003). HBV X protein (HBX), which is critical for viral infection, triggers apoptosis in hepatocytes *in vivo* and *in vitro* independently of p53 (Terradillos et al. 1998). It also sensitises primary mouse hepatocytes to TNF $\alpha$ -induced apoptosis by a caspase 3-dependent mechanism (Kim et al. 2005). Again, however, HBX can act both proapoptotic and anti-apoptotic in primary rat hepatocyte cultures depending on the status of NF- $\kappa$ B (Clippinger et al. 2009). Moreover, HBX can inhibit Fas-mediated cell death in primary human hepatocytes through upregulation of the stress-activated protein kinase (SAPK)/JNK pathway (Diao et al. 2001). In contrast, hepatitis C virus (HCV) infection induces apoptosis in cultured primary human hepatocytes through TRAIL signalling (Lan et al. 2008; Yang et al. 2011). The same outcome is achieved with HCV core protein, and this depends on the activation of protein kinase R (Realdon et al. 2004).

### Miscellaneous induction of hepatocyte apoptosis

In addition to chemical and microbiological agents, a number of other conditions have been described to experimentally induce apoptosis in primary hepatocyte cultures. In this regard, apoptosis can be activated by cultivating hepatocytes in conditioned medium, collected over the first 3 h of cultivation from serum-free rat hepatocyte cultures (Bresgen et al. 2004). Omission of vital chemical factors from the hepatocyte culture medium is another way to evoke cell death, which has been demonstrated for magnesium (Martin et al. 2003) and glucose (Choi et al. 2003). Ischaemia/reperfusion, a condition known to strongly decrease cell survival of liver grafts *in vivo*, can be modelled *in vitro* through a hypoxia/reoxygenation approach (Laurens et al. 2005; Ozaki et al. 2003; Shimizu et al. 1996). Application of this approach to primary hepatocytes leads to caspase 3-mediated apoptosis and involves SAPK/JNK signalling (Crenesse et al. 2003). Similarly, irradiation of primary hepatocytes with ultraviolet light causes p53-dependent apoptosis (Bellamy et al. 1997; Prost et al. 1998; Worner and Schrenk 1996).

### Detection and biomarkers of apoptosis in hepatocyte cultures

While studying apoptosis *in vitro*, *in casu* in primary hepatocyte cultures, a combined battery of general cytotoxicity assays and specific apoptosis detection methods is usually addressed. The former typically consists of classical techniques to assess cell viability. Thus, measurement of plasma membrane integrity can be achieved with dyes that only enter damaged cells, such as trypan blue (Tichy et al. 2010), or dyes that specifically stain vital cells, such as calcein acetoxymethylester (Alvarez et al. 2009). Plasma membrane damage can also be detected through monitoring of the leakage of cytosolic enzymes, such as lactate dehydrogenase, into the extracellular environment (Vinken et al. 2009). At the functional level, cytotoxicity is frequently studied by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, which becomes reduced by mitochondria of living cells to yield a purple formazan product (Hynes et al. 2006).

As regards apoptosis, morphological examination by light microscopy or transmission electron microscopy can reveal the typical features of this type of cell death, including blebbing, cell shrinkage, cytoplasmic and nuclear condensation, and the formation of apoptotic bodies (Kume et al. 2005; Vinken et al. 2009). An early event during the commitment of cells to apoptosis is the externalisation of phosphatidylserine, a negatively charged phospholipid that is normally restricted to the inner surface of the cell plasma membrane bilayer. This process can be monitored by using the phospholipid-binding protein Annexin V, for instance in a flow cytometry setup (Fu et al. 2001). Another crucial process in apoptosis involves the activation of caspases by proteolytic cleavage from their procaspase precursors. This can be studied at the protein level using techniques such as immunoblot analysis, and at the activity level by means of synthetic fluorescent caspase substrates (Vinken et al. 2009). In fact, caspase 3 activation was reported to be a reliable and sensitive marker of apoptosis in primary hepatocyte cultures (Gomez-Lechon et al. 2002). Given their central role in programmed cell death, mitochondria also deliver a number of apoptosis biomarkers. In this respect, subcellular fractionation and assessment of mitochondrial cytochrome C release are common techniques. Furthermore, changes in mitochondrial transmembrane potential can be traced by using lipophilic cationic dyes, such as 3,3-dihexyloxycarbocyanine iodide or rhodamine 123 (Hussain and Frazier 2003). A prototypical hallmark of apoptosis relates to changes in the nucleus. Indeed, DNA fragmentation can be visualised by gel electrophoresis and subsequent ethidium bromide staining (Ribeiro et al. 2010). However, a more popular assay is the terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labelling method, a histochemical technique that allows detection of DNA breaks (Fu et al. 2001).

## Conclusions and perspectives

Since its first description in 1972 (Kerr et al. 1972), apoptosis has been observed in virtually all tissue types both as a mechanism to physiologically remove dysfunctional cells and as a protective response to pathological or toxic insults. Liver apoptosis, mainly occurring in hepatocytes, is particularly relevant in the latter scenario and relies on intrinsic and extrinsic signalling pathways (Gomez-Lechon et al. 2002; Guicciardi and Gores 2005; Malhi and Gores 2008; Malhi et al. 2006; Orrenius et al. 2011; Schattenberg et al. 2006; St-Pierre and Dufour 2012). It should be mentioned, though, that apoptosis is not the sole cell death mode that can be activated in the liver. Besides necrosis (Malhi et al. 2006), autophagy, a cellular self-digestion process which encompasses a highly conserved intracellular catabolic pathway that degrades proteins and organelles, also underlies a multitude of liver diseases (Rautou et al. 2010). Nevertheless, hepatic apoptosis still is a ubiquitous research topic, and this area frequently makes use of *in vitro* test systems. A number of such experimental settings have proven their utility in apoptosis research, such as precision-cut liver slices (Kasper et al. 2005), yet cultures of primary hepatocytes are considered as the gold standard for this purpose. However, they suffer from a number of fundamental disadvantages, which limit their applicability over the long term. Among those are the occurrence of spontaneous apoptosis, triggered during the hepatocyte isolation procedure and resulting in poor cell survival during subsequent cultivation (Elaut et al. 2006; Vinken et al. 2006, 2012b). Conventional strategies to counteract this process typically aim at mimicking the *in vivo*

hepatocyte micro-environment in vitro (Table 1). It is expected that this classical methodological track will be largely pursued in near future. Sophisticated systems, such as tridimensional perfusion bioreactors in which primary hepatocytes are embedded in compartments that are continuously perfused, have been introduced in the last few years. Using this innovative device, it was found that the NO donor *S*-nitrosoglutathione reduces hepatocellular apoptotic cell death (Prince et al. 2010). Although promising, these classical strategies usually tackle the apoptotic response as such without affecting its actual triggers. In recent years, a number of novel approaches have been introduced in this respect. Based on the notion that levels of NO and inducible NO synthase strongly increase during hepatocyte isolation (Elaut et al. 2006), exposure of hepatocytes to the inducible NO synthase inhibitor *l*-omega-nitro-*l*-arginine methyl ester already started during the isolation procedure was found to strongly downregulate caspase activation and apoptosis (Donato et al. 2001). Likewise, addition of the epigenetic modifier trichostatin A to the perfusate used for hepatocyte isolation negatively affects caspase 3 processing as well as the expression of p53, Bid and Bax (Vanhaecke et al. 2006).

Stabilised primary hepatocyte cultures, displaying only a minimal background of spontaneous apoptosis, are valuable in vitro tools for studying liver apoptosis at the mechanistic level, which is not possible in vivo. Experimental induction of apoptosis in primary hepatocyte cultures can be achieved in various ways, involving chemical, microbiological and a number of physical stimuli (Table 2). In turn, the apoptotic response triggered by these noxious factors can be monitored by addressing a number of cytomeric markers, which preferentially cover a combined set of general cytotoxicity parameters and apoptosis-specific read-outs (Table 3). In the last decade, detection of apoptosis in primary hepatocyte cultures at a global scale by using transcriptomics (i.e. DNA micro-arrays) (Kume et al. 2005; Zucchini-Pascal et al. 2011) and proteomics (Rodriguez-Ariza et al. 2005; Rowe et al. 2010) technologies has generated a wealth of new sensitive mechanistic biomarkers. More recently, epigenetic hallmarks of hepatocellular cell death in vitro have also joined into this list (Lei et al. 2010; Vinken et al. 2010). Further exploration of such novel molecular signatures of hepatocellular apoptosis not only is of importance for its accurate and early detection in vitro, but is equally anticipated to yield new in vivo-relevant biomarkers that may be of potential clinical interest.

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## Abbreviations

<b>APAF-1</b>	Apoptotic protease activating factor 1
<b>Bcl-2</b>	B cell lymphoma 2
<b>Caspase(s)</b>	Cysteiny l aspartate-specific proteinase(s)

<b>dATP</b>	Deoxyadenosine triphosphate
<b>DiOC<sub>6</sub></b>	3,3-Dihexyloxacarbocyanine iodide
<b>DMSO</b>	Dimethylsulphoxide
<b>ECM</b>	Extracellular matrix
<b>EGF</b>	Epidermal growth factor
<b>ERK</b>	Extracellular signal-regulated kinase
<b>HBV</b>	Hepatitis B virus
<b>HBX</b>	Hepatitis B virus X protein
<b>HCV</b>	Hepatitis C virus
<b>HGF</b>	Hepatocyte growth factor
<b>IAP</b>	Inhibitor of apoptosis
<b>JNK</b>	c-Jun N-terminal kinase
<b>LDH</b>	Lactate dehydrogenase
<b>LPS</b>	Lipopolysaccharide
<b>MAPK</b>	Mitogen-activated protein kinase
<b>MTT</b>	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
<b>NF-<math>\kappa</math>B</b>	Nuclear factor-kappa beta
<b>NO</b>	Nitric oxide
<b>PI3K</b>	Phosphatidylinositol-3 kinase
<b>SAPK</b>	Stress-activated protein kinase
<b>TGF<math>\beta</math>1</b>	Transforming growth factor $\beta$ 1
<b>TNF<math>\alpha</math></b>	Tumour necrosis factor $\alpha$
<b>TRAIL</b>	Tumour necrosis factor $\alpha$ -related apoptosis-inducing ligand
<b>TUNEL</b>	Terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labelling

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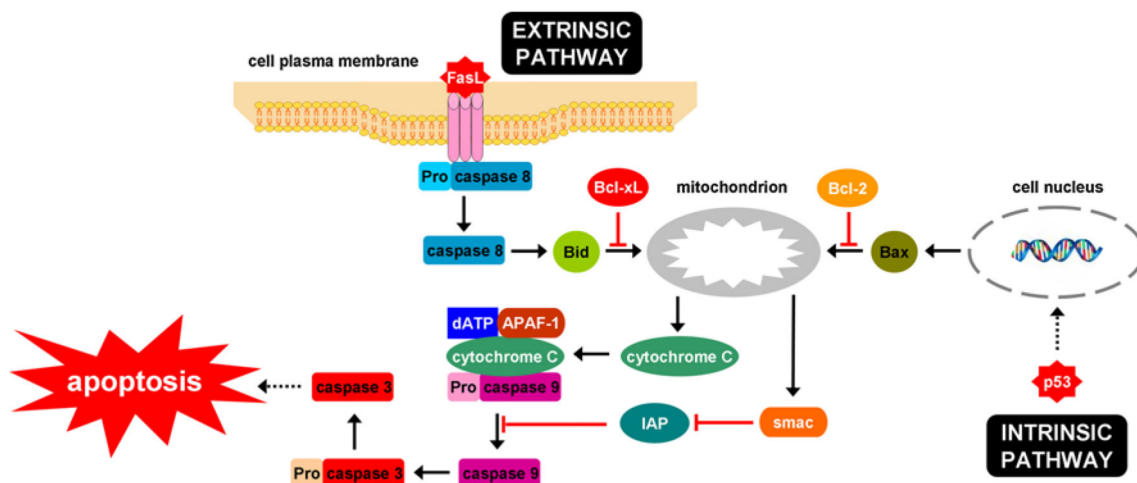
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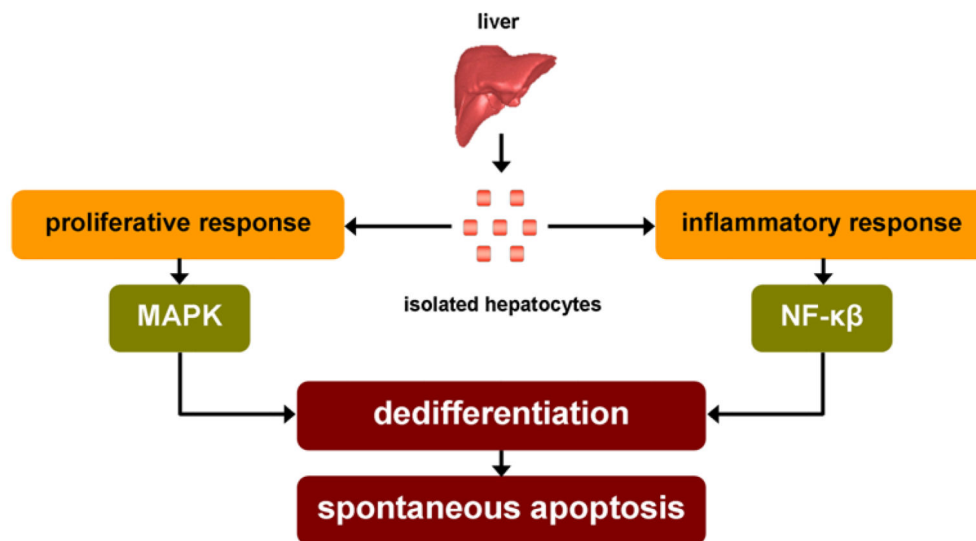
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**Fig. 1.**

Mechanisms of hepatocyte apoptosis. In the intrinsic pathway, which can be activated by p53 upon DNA damage, cytochrome C is released from mitochondria. This process is mediated by B cell lymphoma 2 (*Bcl-2*) proteins which can act either proapoptotic (e.g. *Bid* and *Bax*) or anti-apoptotic (e.g. *Bcl-xL* and *Bcl-2*). Liberated cytochrome C forms an apoptosome with apoptotic protease activating factor 1 (*APAF-1*), deoxyadenosine triphosphate (*dATP*) and procaspase 9. The subsequent activation of caspase 9 can be counteracted by the inhibitor of apoptosis (*IAP*) family, which itself can be blocked by the smac protein. Caspase 9 then triggers caspase 3, which is a major executor of apoptosis by cleavage of a broad spectrum of cellular proteins. In the extrinsic pathway, specific ligands, such as Fas ligand, bind to their corresponding receptors at the cell plasma membrane surface, which induces the recruitment and cleavage of procaspase 8. In hepatocytes, this pathway can as such not lead to activation of caspase 3 and needs mitochondrial amplification, a process that relies on the actions of Bid



**Fig. 2.** Activation of spontaneous apoptosis in hepatocyte cultures. During 2-step collagenase perfusion isolation of hepatocytes from liver tissue, cellular contacts are abolished and ischaemia/reperfusion occurs. These deleterious events trigger an inflammatory reaction, mediated by nuclear factor-kappa beta ( $NF-\kappa\beta$ ), and a proliferative response, mediated by mitogen-activated protein kinase ( $MAPK$ ). In turn, this results in the progressive deterioration of the differentiated phenotype, which ultimately burgeons into the onset of spontaneous apoptosis (adapted from Paine and Andreakos 2004; Vinken et al. 2012b)

**Table 1**

Strategies to counteract spontaneous apoptosis in primary hepatocyte cultures

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<b>The addition of anti-apoptotic molecules to the cell culture medium</b>
EGF/HGF
Insulin/glucagon
Dexamethasone
DMSO
Phenobarbital
Trichostatin A/5-(4-dimethylaminobenzoyl)aminovaleric acid hydroxamide
<b>The restoration of intercellular contacts</b>
Co-cultures with hepatic/non-hepatic partners Spheroid cultures
<b>The re-establishment of an ECM scaffold</b>
Single scaffold cultures on natural/synthetic/non-physiological substrata
Sandwich cultures between natural/non-physiological ECM layers

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*DMSO* dimethylsulphoxide, *ECM* extracellular matrix, *EGF* epidermal growth factor, *HGF* hepatocyte growth factor



**Table 2**

Strategies to experimentally induce controlled apoptosis in primary hepatocyte cultures

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<b>Chemical induction of hepatocyte apoptosis</b>
Fas antibodies/Fas ligand
TNF $\alpha$
TRAIL
TG $\beta$ 1/activin
Hydrophobic bile acids
<b>Microbiological induction of hepatocyte apoptosis</b>
HBV/HBX
HCV/HCV core protein
<b>Miscellaneous induction of hepatocyte apoptosis</b>
Conditioned medium
Omission of vital chemical factors
Hypoxia/reoxygenation
Ultraviolet light irradiation

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*HBV* hepatitis B virus, *HBX* hepatitis B virus X protein, *HCV* hepatitis C virus, *TGF $\beta$ 1* transforming growth factor  $\beta$ 1, *TNF $\alpha$*  tumour necrosis factor  $\alpha$ , *TRAIL* tumour necrosis factor  $\alpha$ -related apoptosis-inducing ligand

**Table 3**

## Detection and biomarkers of apoptosis in primary hepatocyte cultures

<b>Biomarkers</b>	<b>Assays</b>
<b>General cytotoxicity read-outs</b>	
Plasma membrane changes	LDH leakage assay/vital dye exclusion assay
Mitochondrial changes	MTT assay
<b>Apoptosis-specific read-outs</b>	
Morphological changes	Light/transmission electron microscopy
Plasma membrane changes	Annexin V staining/flow cytometry
Cytosolic changes	Caspase 3 activity assay/immunoblot analysis
Mitochondrial changes	Cytochrome C release/DiOC <sub>6</sub> staining
Nuclear changes	Gel electrophoresis/TUNEL assay

*DiOC<sub>6</sub>* 3,3-dihexyloxycarbocyanine iodide, *LDH* lactate dehydrogenase; *MTT* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium, *TUNEL* terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labelling