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An evaluation of the role of insulin-like growth factors (IGF) and of type-I IGF receptor signalling in hepatocarcinogenesis and in the resistance of hepatocarcinoma cells against drug-induced apoptosis

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Abstract

Strong evidence emphasizes the role of the insulin-like growth factor (IGF) system and of type-I IGF receptor (IGF-IR) signalling in tumourigenesis. In this connection: (i) changes in the expression pattern of components of the IGF system (autocrine/paracrine expression of IGF-I and -II, overexpression of IGF-IR, decreased expression of IGF-binding proteins (IGFBPs) and of type-II IGF receptor/cation-independent mannose-6-phosphate receptor (IGF-II/M6PR) and (ii) increased serum concentrations of proteases that cleave the IGFBPs (e.g., cathepsin D) were observed in patients with hepatocellular carcinomas (HCC), in human hepatoma cell lines and in their conditioned culture medium, as well as in rodent models of hepatocarcinogenesis. Accordingly, studies carried out with animal models do suggest that the IGF system and IGF-IR signalling may play a role in hepatocarcinogenesis and in deregulated proliferation and apoptosis of HCC cells. Finally the instrumental role of Raf/MEK/ERK, one of the signalling cascades stimulated by IGF-IR, in anthracycline-induced apoptosis of HepG2 and Huh-7 human hepatoma cell lines emphasizes that care must be taken when designing combinations of antitumoural molecules for antineoplastic treatment. This review addresses the putative roles of the IGF system in primary HCC, with a special focus on the underlying molecular mechanisms. In a second part it emphasizes the putative interference of IGF-IR signalling with chemotherapeutic drug-induced apoptosis in human hepatoma cells.

Keywords: Hepatocarcinogenesis; Hepatocellular carcinoma; Insulin-like growth factors; IGF-binding proteins; Type-I IGF receptor signalling; Apoptosis; Anthracycline antibiotics

1. Introduction

Hepatocellular carcinoma (HCC) accounts for 80–90% of liver cancers and is one of the most frequent carcinomas worldwide. HCC may arise both in liver cirrhosis (60–80% of HCCs) and in non-cirrhotic liver, suggesting that different hepatocarcinogenetic pathways exist. As with other kinds of cancer, the etiology and carcinogenesis of HCC are multi-factorial: infection by hepatitis B and C viruses, cirrhosis of any etiology, primary hemochromatosis, prolonged exposure to mycotoxins such as aflatoxin B1. However, although the precise mechanisms involved may differ according to the risk factor, the multi-step hepatocarcinogenetic process may be divided into: chronic liver injury which produces inflammation, cell death, cirrhosis and regeneration, DNA damage, dysplasia, and finally HCC [1–4].

At the hepatocyte level, hepatocarcinogenesis occurs in distinctly defined stages: ''*initiation*'' which is negatively regulated by the rate of hepatocyte apoptosis, ''*promotion*'' which is characterized by a selective increase in cell proliferation and decrease in apoptosis of preneoplastic hepatocytes, and ''*progression*'' where both cell proliferation and apoptosis appear to be increased [1,2,5–7].

Such an imbalance of the proliferation-apoptosis process may result from the loss of co-ordinated response to

Abbreviations: IGF, insulin-like growth factors; IGF-IR, type-I IGF receptor; IGF-II/M6PR, type-II IGF receptor/cation independent mannose-6-phosphate receptor; IGFBP, IGF-binding protein; ERK, extracellular signal regulated kinase; HCC, hepatocellular carcinoma

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growth factors and cytokines [8–10] among which the insulin-like growth factors (IGF-I and -II) stand as suitable candidates. Indeed, the IGFs are synthesized and secreted in extracellular fluids by foetal as well as adult hepatocytes [11,12]. The interaction of IGF-I and -II with type-I IGF receptor (IGF-IR) plays a pivotal role in the proliferation of a variety of cell types [13–15], in the control of cell cycle progression in G_1 [16], in the regulation of the early phases of tumourigenicity ([17,18]; reviewed in [19,20]), in the maintenance of the tumourigenic phenotype [21–24], and in the prevention of apoptosis [19,20,25–28].

This review will first focus on the putative role the various components of the IGF system may play in the onset of primary hepatocellular carcinomas and consider the underlying molecular mechanisms. In a second part it will emphasize the putative interference of IGF-IR signalling with chemotherapeutic drug-induced apoptosis in hepatoma cells.

2. Endocrine versus paracrine effects of the IGF system

2.1. IGF-I and -II

The parenchymal cells of the adult liver synthesize and secrete IGF-I and -II. However, whereas hepatocytes are the main contributors in liver IGF-I synthesis and secretion [29], hepatocytes as well as resident macrophages (Küpffer cells), endothelial cells and hepatic stellate cells do synthesize IGF-II [30]. Liver IGF-I and -II are secreted in the serum and stand for the *"endocrine"* pool of the IGFs (Fig. 1). On the other hand, both IGF-I and -II are synthesized and secreted in every tissue, by almost every cell-type and play autocrine/paracrine roles (Fig. 1) [11,12].

The *"endocrine"*, growth hormone-dependent, pool of IGF-I has long been thought to be responsible for post-natal growth. However, it has recently been reported that excision of the *Igf1* gene specifically in the liver of transgenic mice (*Cre*/Lox-mediated excision) reduces the circulating concentration of IGF-I by approximately 75%, but does not alter significantly the growth rate of the transgenic animals, when compared to that of wild-type mice [31]. These observations suggest that the IGF-I secreted in extra-hepatic tissues do exert an autocrine/paracrine control on body growth in mammals.

With regard to gene expression, the human *IGF2* gene displays specific characteristics. In all foetal tissues (including the liver) the *IGF2* gene is transcribed from three ubiquitous promoters (P_2–P_4). In contrast, in all the cell types of the adult liver, *IGF2* gene transcription is initiated from a liver-specific promoter (P_1), although ubiquitous promoters P_2–P_4 remain active in adult peripheral tissues [32–34]. Ubiquitous to liver-specific promoter shift in the adult liver is concomitant with a drastic decrease in the abundance of IGF-II transcripts [32].

2.2. IGFBPs

In the serum and in extracellular fluids, IGF-I and -II are bound to six specific, high-affinity, binding proteins. These IGFBPs control IGFs bioavailability and thus IGF-I/IGF-IR interaction at the target cell level [35–38]. As it is the case for the IGFs, two pools of IGFBPs coexist in mammals: synthesized and secreted by the liver (*endocrine*) and

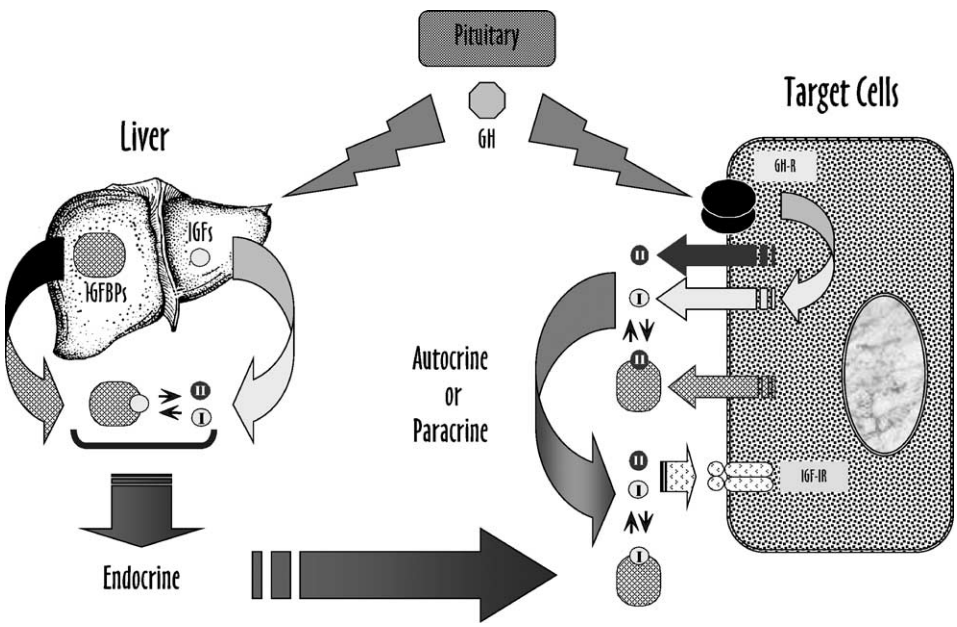


Fig. 1. Schematic diagram of the endocrine vs. autocrine/paracrine effects of the IGFs and IGFBPs.

by peripheral tissues (exerting autocrine/paracrine actions) [35–38] (Fig. 1).

In addition to controlling the pool of bioactive IGFs, the IGFBPs have long been recognized to exert IGF-independent (intrinsic) effects on their own [37,38]. In some cases (IGFBP-3 and -5) the IGF-independent effects might be mediated by cell surface proteins that interact with the IGFBP with high affinity (putative *"receptors"*?) [39–42]. On the other hand, IGFBP-3 has been shown to trigger intracellular signalling: stimulation of phosphotyrosine phosphatase and phosphatidylinositol-3′ kinase (PI-3′ kinase) activities, increase in intracellular calcium [42–44]. Up to date, there is no clearcut evidence that these signalling events are mediated by the interaction of IGFBP-3 with the putative cell surface *"receptor"*. With regard to IGFBP-1, the interaction of the carboxyl-terminal "RGD" motif with the α5β1 integrin receptor has been reported to account for its intrinsic actions on cell migration [45,46].

Finally, both the IGF-dependent and -independent effects of IGFBPs are modulated by limited proteolytic cleavage [37,38,47,48]. Indeed, the release of IGF-I and -II from IGF/IGFBP complexes is best achieved by limited proteolysis of the IGFBPs that generate fragments with reduced affinity for the IGFs ([49,50]; reviewed in [37,38]). In addition, some of the proteolytic fragments do not bind the IGFs at all and may play a role in the inhibition of cell proliferation [51].

Only acid-activated (cathepsins?), but not neutral IGFBP protease activity was detected in primary cultures of rat liver cells [30,52] and accordingly, co-cultures of hepatocytes and of Küpffer cells yielded IGFBP-3 proteolysis [52].

In the liver, IGFBP-1, -2, -4 and the acid labile subunit (ALS, a glycoprotein that do not bind the IGFs directly, but interacts with IGFBP-3 and -5 only when they are associated with IGF-I or -II) are synthesized and secreted by the hepatocytes, whereas IGFBP-3 is only produced by Küpffer, endothelial and hepatic stellate cells [30,53–58]. In the liver, the expression of *Igfbp* and *Als* genes is regulated by several hormones (insulin, glucagon (via cAMP), IGFs). Insulin inhibits *Igfbp-1* and *-2* gene expression in the hepatocytes whereas it stimulates that of *Igfbp-4* and *Als* [57,59,60]. *Igfbp-3* and *Als* gene expression is regulated in a coordinated manner in Küpffer cells and in hepatocytes, respectively: decreased by cAMP [52] and synergistically increased by insulin [57].

2.3. IGF receptors

The expression of the *Igf-1r* gene is very low in hepatocytes whereas significant expression is achieved in Küpffer, endothelial and hepatic stellate cells, suggesting that hepatocytes are poorly responsive to the IGFs whereas Küpffer, endothelial and hepatic stellate cells may be responsive to IGF signalling [61–63]. However, high affinity binding of IGF-II (but not of IGF-I) to type A insulin receptor (IR-A) [64,65] at the hepatocytes plasma membrane might be responsible for triggering an IGF response in the presence of IGF-II.

Finally, every cell type in the liver do express the membrane-bound type 2 IGF/cation-independent mannose-6-phosphate receptor (IGF-II/M6PR) [62,63]. The truncation of the carboxyl-terminal cytoplasmic tail of IGF-II/M6PR yields a soluble form in the serum or in culture medium conditioned with hepatocytes [66,67]. Although the precise physiological relevance of the soluble form of IGF-II/M6PR remains to be determined, it decreases the bioavailability of IGF-II and inhibits IGF-II mediated DNA synthesis [68].

3. Alterations of the IGF system in primary liver cancer

Although the low abundance of IGF-IR in normal, adult hepatocytes (vide supra) suggests only weak IGF effects on liver parenchymal cells in the adult liver, several evidence support the putative contribution of the IGFs, of IGF-II/M6PR and of IGF-IR to deregulated hepatocyte proliferation and/or apoptosis in vivo in the course of hepatocarcinogenesis or in HCCs.

3.1. Increased IGF bioavailability

It may result from at least three complementary and synergistic phenomena: increased IGF production, decrease of both IGF-II/M6PR expression and IGFBP secretion.

3.1.1. IGF-I: paracrine effect on HCC cell proliferation?

A significant reduction of IGF-I serum concentration has been reported in HCC patients, independent of the grade of impairment of liver function [69]. Similarly, the serum level of IGF-I remained unchanged in tumour burdened rats (rats that had developed HCCs in one lobe after inoculation of H_4II rat hepatoma cells), despite enhanced *Igf1* gene expression in non-burdened liver lobes [70]. These observations suggest that IGF-I may play a paracrine role per se and/or cooperate with other cytokine(s) in the course of hepatocarcinogenesis or in HCCs cell proliferation [70].

3.1.2. Increased IGF-II production

A significant 40–100-fold increase in *IGF2* gene expression was observed in human cirrhotic liver, in liver cancers and in human hepatoma cell lines, when compared to that of normal adult liver [71–73]. High focal expression of IGF-II has also been observed in chronic active hepatitis, in persistent hepatitis and in HCCs of hepatitis B and C infected liver [74,75]. In some instances, increased *IGF2* gene expression (i) has been correlated with increased rates of cell mitotic activity, as estimated by proliferating cell nuclear antigen (PCNA) expression [76]

and (ii) may contribute to tumoural angiogenesis [77]. Similarly, *Igf2* gene expression was reactivated during hepatocarcinogenesis in transgenic mice and was associated with high replicative activity, but not with changes in apoptosis [78,79].

Interestingly, re-expression and overexpression of the *Igf2* gene in mouse and human HCCs, respectively, was concomitant (i) with the re-activation of a foetal pattern of gene expression [71,72,76] and (ii) with silencing of the liver-specific promoter P_1 in human HCCs [80]. In addition, overexpression of the *IGF2* gene in human preneoplastic foci and HCCs has also been reported to be associated with the restoration of an allelic imbalance at the *IGF2* locus [81,82].

That overexpression of IGF-II may be involved in the hepatocarcinogenetic process, or in HCC cell proliferation could be deduced from ex vivo experiments. HuH-7 and HepG2 human hepatoma cells produced five-fold more intracellular IGF-II than other cell lines. When the production of IGF-II was suppressed by specific antisense oligodeoxynucleotides, the decrease in IGF-II peptide resulted in growth inhibition of HuH-7 and HepG2 cells [83]. Accordingly, we have shown that IGF-I-stimulated proliferation of HepG2 cells is enhanced when endogenous IGF-II production has previously been suppressed, targeting IGF-II transcripts with small interfering RNA (Alexia et al., unpublished observation).

3.1.2.1. Molecular mechanisms that underlie reactivation of a foetal pattern of IGF2 gene expression in preneoplastic foci and in HCCs. On the one hand, an elegant study by Eriksson et al. [84] has evidenced a close correlation between *IGF2* gene expression from P_3 and the methylation status of the promoter, with expression being linked to promoter hypomethylation. On the other hand, the hepatitis B virus X protein (HBx) has been shown to up-regulate *IGF2* gene expression from P_4 via increased Sp1 (or Egr-1 or -2) binding to bona fide Sp1 *cis*-elements [85]. HBx does not activate Sp1 through direct protein–protein interaction, but rather via an indirect mechanism that requires the activation of both protein kinase C (PKC) and ERK-1/ERK-2 signalling [86]. Interestingly, the protein phosphatase activity of the tumour suppressor gene PTEN (phosphatase and tensin homologue on chromosome 10)/MMAC1/TEP1 blocks Sp1 phosphorylation in response to HBx, by inactivating PKC and ERK-1/ERK-2 activities [87]. In this connection, it should be stressed that PTEN is frequently inactivated by mutation or deleted in human HCC (vide infra).

Finally in some cases, accumulation of IGF-II in HCCs tissue could be due to up-regulation of *IGF2* gene transcription by p53mt249, a gain-of-function mutant of p53 frequently observed in patients that have developed HCCs after prolonged exposure to aflatoxin B1 [88]. p53mt249 enhances transcription from the foetal IGF-II promoter P_4 [88].

3.1.3. Loss of IGF-II/M6PR function

Loss of heterozygosity (LOH) and mutations at the *IGF-2R* locus have been reported in HCCs and in adjacent liver tissue [89–92], and compelling genetic evidence has been provided that loss of the *IGF-2/M6PR* gene is an early transformational event that occurs frequently in human HCCs ([93]; reviewed in [94]). Accordingly, no IGF-II/M6PR was detected in preneoplastic liver lesions induced in rodents by chemical carcinogens [95].

One function of IGF-II/M6PR consists in tissue degradation of IGF-II (reviewed in [94]). Thus, LOH and/or inactivating mutations of the IGF-II binding site at the *IGF-2/M6PR* locus yields increased IGF-II bioavailability in the extracellular fluids and allows one to predict enhanced proliferation and decreased apoptosis of HCC cells through activation of IGF-IR and/or IR-A signalling. These molecular events favour HCC progression and may synergize with other alterations related to LOH and/or inactivating mutations at the *IGF-2/M6PR* locus (decreased activation of latent TGFβ, increased secretion of proteolytic enzymes) that reinforce cellular proliferation and enhance local invasion and metastasis, suggesting tumour suppressor roles for the *IGF-2/M6PR* gene [94].

Accordingly, suppression of both IGF-II/M6PR and IGF-IR gene expression in human hepatoma cells depressed their growth capability in soft agar and their tumourigenicity in nude mice [96].

3.1.4. Decreased IGFBP secretion

IGFBP-1, *-3* and *-4* gene expression is unaltered in cirrhotic liver, but down-regulated in surgical specimens of human HCC tissues [97]. Specifically, IGFBP-3 levels are low or undetectable in human HCC samples [98] and IGFBP-1 and -2 are decreased in hepatoblastoma [99], when compared to non-neoplastic liver tissue. These observations suggest increased IGF-I and -II bioavailability in the serum and in extracellular fluids and allow one to predict enhanced proliferation and decreased apoptosis of HCC cells through activation of IGF-IR signalling.

Accordingly, the addition of exogenous IGFBP-1 to -4 to the human hepatoma cell line PLC or of IGFBP-3 to HepG2 cells leads to significant reduction in cell proliferation [98,100]. In the latter case, co-treatment of HepG2 cells with IGFBP-3 and IGF-I attenuated IGF-IR signalling (e.g., Raf/MEK/ERK-1/ERK-2, PI-3′ kinase) and the mitogenic activity of IGF-I [98].

Moreover, the decreased *IGFBP* gene expression in HCC tissue may otherwise counteract their intrinsic, IGF-independent effects. This stands particularly true for the inhibitory effect of IGFBP-3, on cell proliferation ([49]; reviewed in [101]). In addition, low *IGFBP-3* gene expression should decrease IGFBP-3-dependent activation of a phosphotyrosine phosphatase and thus relief the subsequent blockade of IGF-dependent IGF-IR signalling [43].

3.1.4.1. Molecular mechanisms that underlie decreased IGFBP gene expression in HCC cells. In human HCC tissue, the decreased *IGFBP-3* gene expression may be related to tumour specific promoter hypermethylation [102] a hallmark of gene silencing. On the other hand, suppression of IGFBP-1 protein in HCC tissue may result from activation of IGF-IR signalling (constitutive or due to autocrine/paracrine IGF-II production; vide supra). Indeed, Lee et al. [103] have shown that decreased secretion of IGFBP-1 by HepG2 cells treated with IGF-I was accompanied by decreased IGFBP-1 mRNA levels and by a significant inhibition of IGFBP-1 promoter activity. A similar mechanism may apply to the decrease in IGFBP-2 production in HCC tissue since its gene expression is inhibited by insulin signalling [104].

Finally it must be stressed that, in addition to altering quantitative *IGFBP* gene expression, hepatocarcinogenesis may deregulate its cell-specificity. For instance, *IGFBP-3* gene expression which is restricted to Küpffer, endothelial and hepatic stellate cells in normal liver tissue extends to hepatocytes in human HCC cells and is found in human hepatoma cell lines (AG, unpublished observations).

3.1.5. IGFBP protease activity

Since IGFBP proteolysis favours the dissociation of IGF/IGFBP complexes in the extracellular fluids and thus increases IGF-IR activation, it is believed to contribute to carcinogenesis. However, although (i) different proteases have been detected in HCC tissue and (ii) increased cathepsin D levels were observed in the serum of HCC patients [105], the role of these proteases in hepatocarcinogenesis remains to be established (see [106] for a comprehensive review on the actions of IGFBP on epithelial cancer cells).

A convincing study by Martin et al. [107] brings about evidence for a role of IGFBP proteolysis in liver tumour development. In SV40Tag and tissue inhibitor of metalloproteinase-1 (TIMP-1) double transgenic mice, TIMP-1 blocks liver hyperplasia, despite Tag-mediated reactivation of *Igf2* gene expression. IGFBP-3 protein levels were elevated and IGFBP-3 proteolysis was significantly lower in TIMP-1-overexpressing animals, lowering the levels of bioavailable (free) IGF-II. These events resulted in diminished IGF-I receptor signalling in vivo as evidenced by diminished receptor kinase activity and decreased tyrosine phosphorylation of IGF-IR downstream effectors (e.g., IRS-1, ERK-1/ERK-2).

3.2. Activation of signalling cascades

3.2.1. Increased igf-1r gene expression

A growing body of evidence suggests that under physiological conditions, *IGF-IR* gene transcription is maintained under inhibitory control by negative growth regulators and/or tumour suppressor gene products and that cells with a reduced number of cell surface receptors (e.g., normal hepatocytes) are unable to progress through the cell cycle (i.e., remain in a post-mitotic state) (reviewed in [108]).

Increased *IGF-IR* gene expression was observed in human cirrhotic liver, in liver cancers and in human hepatoma cells, when compared to that of normal adult liver [61,100,109,110]. Up-regulation of *IGF-IR* gene transcription by p53mt249 (vide supra), could account for increased *IGF-IR* gene expression in some HCCs [111]. Similarly, *IGF-IR* gene expression is increased in HCCs and in hepatoma cell lines that express HBx [112,113].

Accordingly, the higher level of IGF-IR in HBx-expressing hepatoma cells and in hepatoma cells expressing p53mt249 enhances the mitogenic effect of IGF-I and -II [100,111,112].

3.2.2. Constitutive activation of signalling cascades

Insulin receptor substrate-1 (IRS-1) an adapter molecule involved in IGF-IR-mediated signal transduction is overexpressed in human HCCs, but not in adjacent, non-tumoural liver [114,115]. That IRS-1 overexpression in HCC tissue may lead to constitutive activation of downstream signalling cascades has been demonstrated in animal model systems. Indeed, targeting the overexpression of IRS-1 in the liver of transgenic mice yields constitutive activation of the Raf/MEK/ERK-1/ERK-2 and PI-3′ kinase signalling cascades and lead to increased DNA synthesis [114]. In addition, overexpression of IRS-1 in transfected human hepatoma cells protects the cells against apoptosis [116].

Our investigations on the role of the Raf/MEK/ERK-1/ERK-2 signalling cascade on chemotherapeutic drug-induced apoptosis have pointed out a constitutive activation of this signalling cascade in Huh-7, but not in HepG2 human hepatoma cells (vide infra), pointing out the heterogeneity of HCC cells with regard to the activation status of the signal transduction pathways.

4. Genomic alterations of IGF signalling in hepatocarcinomas

Several evidence support the predominant role of PI-3′K signalling in hepatocyte [117] and hepatoma cell proliferation (Alexia et al., submitted for publication), and suggest that up-regulation of PI-3′ kinase/Akt signalling may contribute to the progression of preneoplastic lesions as HCCs. Interestingly, some genetic alterations detected in HCC patients may account for constitutive activation of the PI-3′ kinase signalling cascade in HCC and in hepatoma cells.

First, inactivating mutations, but also LOH of *PTEN*, a tumour-suppressor gene which is located on 10q23.3, have been reported in approximately 10% of HCCs, resulting in constitutive activation of the PI-3′K signalling [118–121].

Second, glycogen synthase kinase-3β (GSK-3β), a downstream target of PI-3′ kinase/Akt signalling, controls β-catenin phosphorylation and regulates the activity of the Wnt/β-catenin pathway. Activation of this pathway can be caused by activating mutations of *β-CATENIN* or by inactivating mutations of the *AXIN* gene [122]. Somatic mutations of β-catenin have been observed in HCCs. These mutations lead to nuclear accumulation of the aberrant protein and activate other transcription factor (e.g., LEF/TCF) [123–126]. Axin is also mutated in approximately 10% of HCCs and lead to the activation of the Wnt pathway [127]. Accordingly, virus-mediated transfer of the *AXIN* gene induces apoptosis in HCC cells, suggesting that axin inhibits growth of hepatocytes [127]. In this connection, inhibition of GSK-3β by Akt stimulates LEF/TCF transcription in HepG2 cells [128].

5. Interference of IGF-IR signalling with chemotherapeutic drug-induced apoptosis of hepatoma cells

5.1. Mechanisms of action of anthracycline antibiotics

The mechanisms of action proposed for antiproliferative and cytostatic effects of anthracycline antibiotics such as doxorubicin include (i) the interference with DNA biosynthesis in tumour cells related either to DNA intercalation or inhibition of DNA polymerase activity [129,130], (ii) generation of free radicals [131,132], (iii) DNA adducts formation and DNA cross-linking [133], (iv) interference with DNA helicase [134], (v) induction of DNA damage via interference with topoisomerase-II activity [135] and (vi) interaction with plasma membrane components [136–138].

On the other hand, the importance of stress signalling in apoptosis triggered by anthracycline antibiotics has been extensively studied over the past decades (reviewed in [139]). Specifically, the importance (i) of Jun NH2-terminal kinase (JNK) [140,141] and AP-1-mediated transcriptional activation [142], (ii) of the tumour suppressor gene p53 [143,144], (iii) of the sphingolipid-derived second messenger ceramide [145–148] and, depending on the cell type and cellular context, (iv) of NFκB signalling [149–152] has been emphasized. In some cases, alterations of endoplasmic reticulum (ER) homeostasis (e.g., protein folding or ER overload) may contribute to cell death [153,154].

With regard to human hepatoma cell lines (e.g., HepG2, Huh-7, Hep3B, SK-Hep-1, PLC/PRF/5) it has been reported that chemotherapeutic drugs can induce apoptosis via Fas-dependent and -independent pathways [155], depending on the p53 status of the cell. Up-regulation of CD95/Fas receptor expression was only observed in cells with wild-type p53 (HepG2), not in cells with mutated p53 (Huh-7) or devoid of p53 (Hep3B) [156]. The mechanisms underlying apoptosis have also been shown to be dependent on the drug used. In HepG2 cells, high doses of cisplatin up-regulate *p53* and $p21^{cip1}$ gene expression before apoptosis occurs [157], whereas induction of apoptosis by high doses of doxorubicin is concomitant with down-regulated $p21^{cip1}$ gene expression [158].

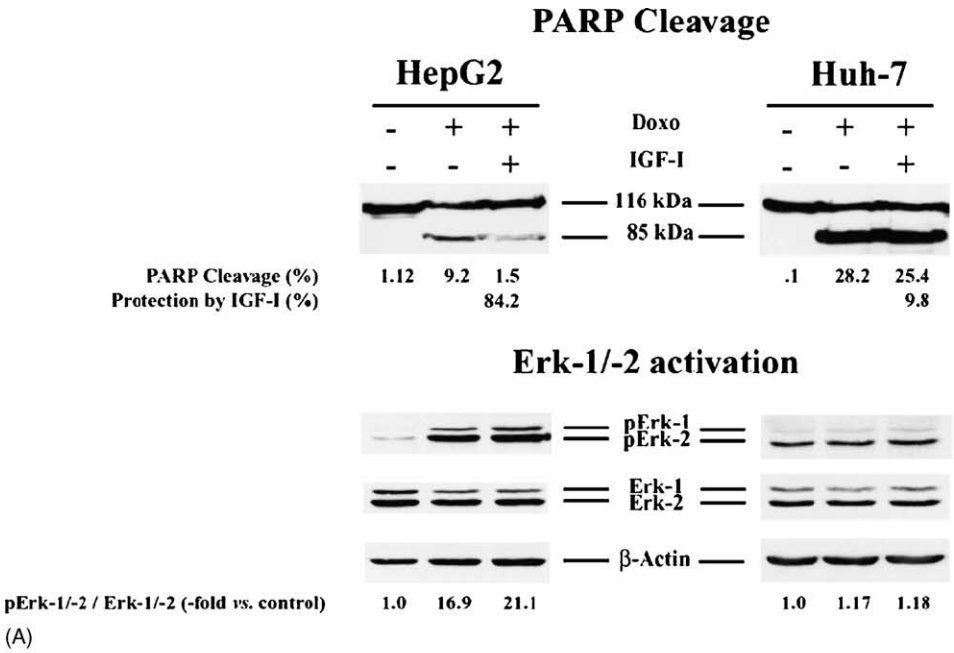
5.2. ERK-1/ERK-2 signalling is instrumental in drug-induced HepG2 and Huh-7 cells apoptosis

In order to get a deeper insight in the mechanisms underlying drug-induced apoptosis in human hepatoma cells, we have examined whether or not doxorubicin (or cisplatin) interfered with the two major signalling cascades stimulated by IGF-IR (PI-3′ kinase and Raf/MEK1/ERK-1/ERK-2). Two cell lines were used as model systems (HepG2 and Huh-7).

As expected, the cytostatic drug doxorubicin triggered apoptosis of HepG2 and Huh-7 cells, as monitored by DAPI staining, flow cytometry (not shown) and PARP cleavage (Fig. 2), with Huh-7 cells being more sensitive to doxorubicin treatment than HepG2 cells [158]. Apoptosis was almost completely reversed in HepG2 cells (84%), but not in Huh-7 cells (<10%) after combined doxorubicin and IGF-I treatment (Fig. 2A, upper panel). Strictly identical results were obtained when HepG2 and Huh-7 cells were treated with cisplatin, another antitumoural agent (not shown). The inability of IGF-I to protect Huh-7 cells against doxorubicin-induced apoptosis was due to the very low level of IGF-IR at the plasma membrane (Alexia et al., unpublished observations). Such heterogeneous levels of IGF-IR gene expression may either mirror intrinsic heterogeneity among HCCs or result from secondary loss of IGF-IR expression in some HCCs.

The treatment of HepG2 or Huh-7 cells with doxorubicin did not alter the level of ERK-1/ERK-2 when compared to that of the housekeeping protein β-actin, suggesting that apoptosis was not dependent on ERK-1/ERK-2 proteolytic cleavage. Moreover, studying the activation status of the ERKs, we have observed that ERK-1/ERK-2 phosphorylation was significantly increased (∼17-fold) after exposure of HepG2 cells to doxorubicin (Fig. 2A, lower left panel). Combined doxorubicin and IGF-I treatment further increased ERK-1/ERK-2 activation by 30%. In contrast, constitutive ERK-1/ERK-2 phosphorylation was not enhanced when Huh-7 cells were treated either with doxorubicin or with doxorubicin plus IGF-I (Fig. 2A, lower right panel).

Whether Raf/MEK1/ERK-1/ERK-2 signalling was instrumental in drug-induced apoptosis of HepG2 and Huh-7 cells was addressed using a specific MEK-1 inhibitor (PD98059). The addition of PD98059 alone to the culture medium of proliferating HepG2 cells did not induce apoptosis, as estimated by the PARP cleavage assay (Fig. 2B). In contrast, doxorubicin-induced PARP cleavage (13-fold when compared to that of untreated cells) was



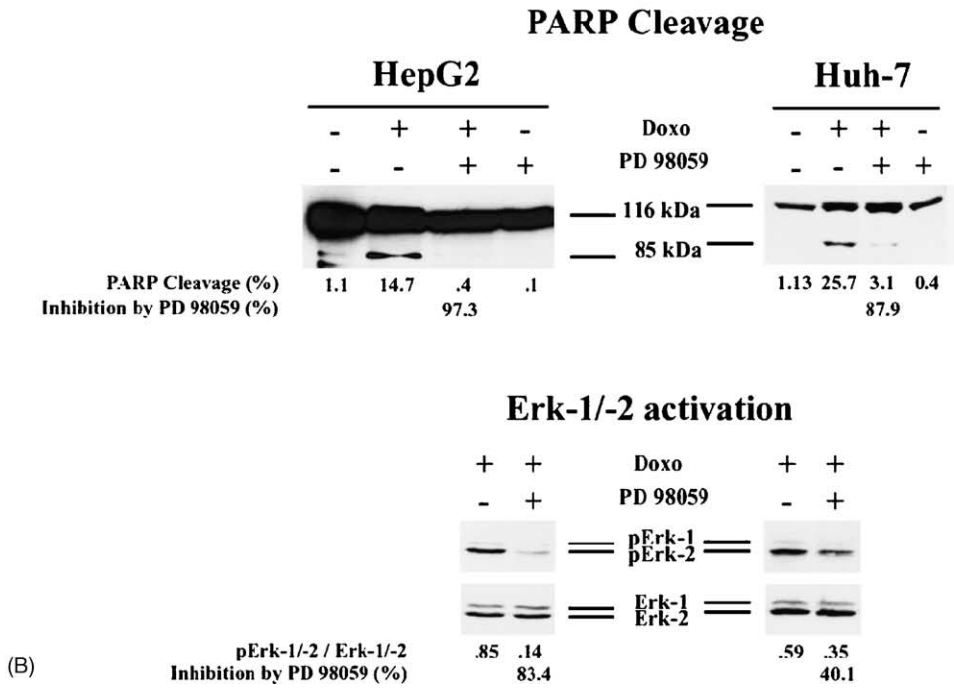


Fig. 2. IGF-I protects HepG2 but not of Huh-7 human hepatoma cells from drug-induced apoptosis: instrumental role of ERK-1/ERK-2 signalling. Monolayers of HepG2 and Huh-7 cells were left untreated (control) or treated with (A) 4 μg ml^{-1} (HepG2) or 2 μg ml^{-1} (Huh-7) doxorubicin ±14 nM IGF-I, or (B) either with 50 μM PD 98059 alone, or with 4 μg ml^{-1} (HepG2) or 2 μg ml^{-1} (Huh-7) doxorubicin ±50 μM PD 98059. Whole cell extracts (lower panels) or nuclear extracts (upper panels) were prepared and analysed by western blot using antiPARP (upper panels) or antiphospho-ERK-1/ERK-2 or antipan ERK or β-actin (lower panels). Western blots were analysed by laser densitometry scanning, then the percentage of cleaved PARP (upper panels) or the pERK/ERK ratios (lower panels) were calculated in each case. In part (A) the pERK/ERK ratios of doxorubicin ± IGF-I-treated cells were computed relative to that of control cells.

almost completely blunted (97% inhibition) by the doxorubicin and PD98059 combined treatment. Similarly, PD98059 inhibited doxorubicin-induced PARP cleavage by 88% in Huh-7 cells (Fig. 2B, upper panel). Strictly identical results were obtained when HepG2 and Huh-7 cells were treated with cisplatin (not shown). As expected, ERK-1/ERK-2 phosphorylation was inhibited by more than 80% when HepG2 cells were treated with both doxorubicin and PD98059 (Fig. 2B, lower panel). Similarly, constitutive ERK-1/ERK-2 activity was inhibited, albeit at a lower level (40%), in Huh-7 cells treated with doxorubicin and PD98059 (Fig. 2B, lower panel).

Finally, our data show that although IGF-I further enhances ERK activation in doxorubicin-treated HepG2 cells, it protects these cells against doxorubicin-induced apoptosis. This may be explained by the simultaneous activation of Raf/MEK-1/-2/ERK-1/ERK-2 and PI-3′ kinase signalling cascades after IGF-I treatment, and by the dominant effect of the latter in the protection of HepG2 cells against apoptosis. This hypothesis was supported by the lack of protection against doxorubicin-induced apoptosis of HepG2 cells co-treated with IGF-I and LY294002, a specific inhibitor of PI-3′ kinase (Alexia et al. manuscript in preparation).

5.3. Raf/MEK-1/-2/ERK-1/ERK-2 signalling is not sufficient for drug-induced apoptosis to occur in HepG2 and Huh-7 cells

Activation of Raf/MEK-1/-2/ERK-1/ERK-2 signalling per se do not appear to be sufficient to trigger apoptosis (reviewed in [159,160]). Indeed, ERK-1/ERK-2 are constitutively activated in several tumours including hepatocarcinoma [161,162] and many studies support the general view that activation of the ERK cascade transduces survival signals [163–165], including in HCCs and hepatoma cell lines [166,167]. Accordingly, Mitsui et al. [165] reported that apoptosis is induced by serum deprivation in HepG2 cells and that MEK1/ERK signalling is required for the cells to survive in the presence of serum. Similarly, ERK activation in HepG2 cells treated with IGF-I (vide supra) or in HeLa cultured in the presence of TPA [168] do not induce apoptosis. In addition, inhibition of ERK signalling has been shown to lead to increased sensitivity to cisplatin [169,170] and to enhance paclitaxel-induced apoptosis in human breast, lung and ovarian carcinoma cell lines [171].

In contrast, our data show that doxorubicin-induced (HepG2) or constitutive (Huh-7) ERK-1/ERK-2 activity is necessary for apoptosis to occur in doxorubicin- and cisplatin-treated cells. Since apoptosis was inhibited when the cells were co-treated with doxorubicin and the MEK-1 inhibitor PD98059, doxorubicin-induced apoptosis probably depends on activation of the Raf/MEK-1/MEK-2/ERK-1/ERK-2 signalling cascade. Similarly activation of ERK-1/ERK-2 signalling has been reported (i) to be important in cisplatin-induced apoptosis of HeLa and human lung A549 cells [168], (ii) to enhance c-Fos-mediated cell death in immortalized murine hepatocytes [172]. In this connection, Fokstuen et al. [173] have reported that inhibition of Ras function decreases the sensitivity of human melanoma cells to cisplatin-induced cell death, and Viktorsson et al. [174] have demonstrated that H-ras-G12V-transformed FR3T3 fibroblasts are more sensitive to cisplatin treatment than the parental cell line.

Several pathways could synergize with ERK-1/ERK-2 to account for apoptosis in hepatoma cells treated with antineoplastic drugs: (i) induction of CD95/Fas receptor and of Fas-ligand (Fas_L) in cells that express wild-type p53 (HepG2; [175]), (ii) cleavage and inactivation of Akt, a kinase with antiapoptotic properties downstream in the PI-3′ kinase signalling cascade, as already observed in human monocytic leukemia (U397) and T (Jurkat) cell lines [176], (iii) activation of the MKK4/7/JNK cascade [140,141] and subsequent AP-1-mediated activation of Fas_L promoter activity (HepG2; [142]). In contrast, the increased sensitivity of H-ras-G12V-transformed FR3T3 fibroblasts to cisplatin was correlated with a weaker activation of JNK by the drug [174] and such a negative cross-talk relationship between the JNK and ERK pathways was also observed in Cos-7 cells transfected with the mixed lineage kinase (i.e., that displays both serine/threonine and tyrosine kinase activities) MLK3 [177].

A cDNA array analysis of the cytotoxic response elicited by cisplatin in p53-deficient Hep3B human hepatoma cells has pinpointed a series of genes the induction of which may contribute to apoptosis [178]. Some of these genes (IGFBP-3, IGF-II/M6PR) are members of the IGF system that display reduced expression or inactivating mutations in human hepatocarcinomas (vide supra) [102,179]. Others are (i) pro-apoptotic members of the Bcl2 family (Bax, Bak), (ii) an inducer of apoptosis, Siva, that interacts with CD27, a member of the tumour necrosis factor receptor family [180], (iii) Raf-1, an upstream serine/threonine protein kinase in ERK-1/ERK-2 signalling, (iv) genes regulating the progression through the G_1 phase of the cell cycle or facilitate the G_1/S transition (cyclin D_1, Cdk-4, -6), consistent with the observation that G_1 arrest was not observed in Hep3B cells exposed to high doses of cisplatin [157]. In this connection, the generation of conflicting signals (i.e., induction of pro-apoptotic genes and of genes involved in cell cycle progression) in cells treated with chemotherapeutic drugs may lead to apoptosis.

In conclusion, one must keep in mind that not only the tumour heterogeneity, even for a given tissue (e.g., HepG2, Huh-7 and Hep3B hepatoma cells differ at least in their p53, CD95/Fas receptor and IGF-IR status) ([155,156]; Alexia et al., unpublished observations), but also the differential response to various chemotherapeutic agents must be taken into account when designing any antineoplastic treatment. As a matter of fact, it has been reported that JNK, ERK and p53 play distinct roles in doxorubicin-, etoposide- and vinblastin-mediated apoptosis of KB-3 carcinoma cells [181].

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