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Original article

Characterization of cell death events induced by anti-neoplastic drugs cisplatin, paclitaxel and 5-fluorouracil on human hepatoma cell lines: Possible mechanisms of cell resistance

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Abstract

Two different hepatoma cell lines were incubated for 48 h with chemotherapeutic drugs cisplatin, paclitaxel and 5-FU to determine their ability to induce cytotoxicity and DNA fragmentation as well as to modify the expression of some cell death-related genes that could be involved in the resistance to therapy. We observed that cisplatin and paclitaxel induced cytotoxicity, but significant differences between both cell lines, were found only in the case of paclitaxel. At 48 h, apoptosis was clearly present in Hep3B cells treated with cisplatin and HepG2 cells treated with paclitaxel. 5-FU induced cytotoxicity in both cell lines but only at higher concentrations than the other two drugs, triggering apoptosis and necrosis in HepG2 cells and only necrosis in Hep3B. When a time course was performed for the first 8 h of treatment to elucidate the initial mechanism of cell death responsible for DNA fragmentation, we observed that 5-FU in Hep3B, and cisplatin in both cell lines, induces primary necrosis, whereas at the concentration tested here, paclitaxel clearly triggers apoptosis in both cell lines. HepG2 cells were weakly sensitive to 5-FU in the first 8 h of treatment, so the primary mechanism of cell death was not clear, but results seem to indicate that it could be apoptosis. At 48 h, Bax was not up-regulated with any of the treatments, whereas cisplatin was able to induce Bcl-xL down-regulation in both cell lines. Treatment with 5-FU also down-regulated Bcl-xL in HepG2 cells. We also measured variations in the expression of survivin, an inhibitor of apoptosis that has also been involved in mitototic catastrophe. Hep3B cells seem to show an increase in protein levels with all treatments. Exposure to paclitaxel resulted in the highest effect. In the case of HepG2 cells, there was a decrease in survivin expression when cells were treated with 5FU and paclitaxel, both treatments showing complete loss of the protein. Using an antibody that recognizes unprocessed caspase-3, we observed that the enzyme was assumingly activated in HepG2 cells treated with 5FU and paclitaxel, but only weakly after treatment with cisplatin. Hep3B cells did not show activation since the levels of the pro-enzyme remained the same as that in the control. In conclusion, the three drugs tested in this study could induce cell death, with paclitaxel being more effective inducing apoptosis. 5FU was only effective at high doses and its mechanism seems to be primarily related to necrosis in Hep3B and probably apoptosis in HepG2. Cisplatin mechanism of cell death is probably mediated by the decrease in anti-apoptotic protein Bcl-xL whereas paclitaxel and 5FU are decreasing the apoptosis inhibitor survivin. According to pro-enzyme levels, caspase-3 was only activated in HepG2 cells, whereas in the case of Hep3B cells the mechanisms of toxicity appear to be caspase-3-independent at the time and concentrations tested in this study. The resistance of Hep3B cells to death induced by

Abbreviations: 5-FU, 5-flurouracil; MTT, 3-(4,5-dimethyl-2-thiazolyl(-2,5-diphenyl-2*H*-tetrazolium bromide); ELISA, enzyme-linked immunosorbent assay; BrdU, 5′-bromo-2′-deoxy-uridine; HCC, hepatocellular carcinoma; SDS-PAGE, sodium sulfate-polyacrylamide gel electrophoresis; IgG, immunoglobulin G; GAPDH, glyceraldehyde-3-phosphate dehydrogenase, 4HPR, *N*-(4-hydroxyphenyl) retinamide; PBS, phosphate buffered saline solution, IAP, inhibitor of apoptosis.

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chemotherapy could be related to an increase in the expression of IAP survivin, which can decrease cell response to the treatment or even switch the type of death from apoptosis to another kind, making therapy less efficient.

Keywords: Cancer; Hepatocellular carcinoma; Chemotherapy; Apoptosis; Survivin

1. Introduction

It is commonly assumed that apoptosis is the mechanism that mediates the actions of cytotoxic drugs and ionizing radiation in tumors [1]. However, conclusive data is lacking and there is evidence that the mechanisms of apoptosis are more diverse than we previously thought and several new forms of cell death have been proposed [2–4]. Also, even regarding the drugs that have been extensively proven to induce apoptosis in some cell types, the exact mechanisms are not completely clear and more information is needed to individualize the tumors regarding their gene defects to improve therapy based on their characteristics. Resistance to chemotherapeutic drugs due to the expression or lack of expression of different genes is a problem in cancer treatment and to overcome this, clinical resistance has become one of the most important goals in therapy [5].

The most frequent type of primary liver cancer is hepatocellular carcinoma (HCC), which is a tumor very resistant to drug therapy [6]. Cell lines derived from these tumors, such as Hep3B, have been shown to have mutations in genome guardian gene p53 [7,8], making them highly insensitive to drugs that act through p53-dependent apoptosis pathways. The opposite is observed in another cell line derived from this type of tumor, HepG2 cells. These cells have no reported mutations in p53 [9] and being, at least in this aspect, close to normal hepatocytes, makes them a useful model to study the effects of chemotherapeutic drugs in liver cells.

Several studies have shown that anti-apoptotic protein Bcl-2 is not expressed in hepatocytes [10,11], but its relative Bcl-xL, is present in both wt-p53-positive and p53-negative hepatoma cell lines. Several authors have established the role of Bcl-xL in hepatic cells under stress [12,13]. Luo and collaborators [13], for instance, showed that a decrease in the expression of this anti-apoptotic protein rendered HepG2 cells susceptible to apoptosis induced by paclitaxel and doxorubicin. Interestingly, ectopic expression of active p53 in Hep3B cells was not enough to induce apoptosis, but when Bcl-xL was simultaneously inhibited by antisense oligonucleotides, apoptosis occurred [12]. These results suggest that Bcl-xL may function as an inhibitor of p53-activated cell death and perhaps other apoptotic pathways induced by chemotherapeutic drugs in HCC.

Another very important physiological contributor of apoptosis is the system of Fas and its ligand (FasL) [14]. A very interesting paper by Jiang and collaborators [15] showed that Fas could be involved in the cytotoxic effects induced by 5-fluorouracil (5-FU) and cisplatin on HepG2 cells, but not in Hep3B. In this study HepG2, but not Hep3B, increased the expression of Fas when treated with these chemotherapeutic drugs. Also, agonistic and antagonistic anti-Fas antibodies triggered and blocked drug-induced apoptosis in HepG2 cells, respectively [15].

Regarding the apoptotic events induced by clinically used drugs in liver tumors, Müller et al. [16] provided the evidence that linked p53 and the Fas-FasL system. They not only showed that cisplatin and other chemotherapeutic drugs up-regulated both Fas and FasL only in wild type-p53 cells such as HepG2, but they also demonstrated that wt-p53 binds and transactivates Fas gene through p53-responsive elements in its promoter [16]. These observations support the hypothesis that one of the mechanisms by which anti-neoplastic drugs can induce apoptosis in a p53-dependent manner in liver cells is by over-expression of Fas, which could also explain drug resistance observed in p53-lacking tumors and transformed cell lines such as Hep3B.

It is very important to point out that p53-lacking cells do undergo apoptosis when treated with some drugs, leading to the possibility that several other mechanisms may be taking place that remain to be identified. An example of this effect was demonstrated in an study by You and collaborators [17] where it was shown that a synthetic retinoid, *n*-(4-hydroxyphenyl) retinamide (4HPR) was able to induce apoptosis in a higher extent in Hep3B cells than in HepG2 cells, strengthening the idea that neither p53 nor Fas were necessary for the toxicity mediated by this drug. 4HPR did not affect Bax protein levels, whereas Bcl-xL levels suffered a small but clear decrease after 72 h of treatment [17]. Our results with naturally occurring retinoids also support the idea that Hep3B cells are more susceptible than HepG2 to the treatment with these retinoids, and that apoptosis can be triggered independently of p53 [18].

Survivin is an anti-apoptotic protein expressed in almost all common cancers including HCC. Some studies have established a correlation between survivin expression and the stage of progression of HCC [19] and others have shown that survivin expression is inversely associated with apoptosis in the same type of tumor [20]. Moreover, when survivin is silenced with antisense oligonucleotides, an increase in apoptotic index has been observed in HepG2 cells [21], indicating that this protein could be involved in the apoptotic events that occur in HCC exposed to chemotherapy.

Cisplatin, paclitaxel and 5-FU are three very different and widely used anticancer agents with a broad range of antitumor activities. The pyrimidine antimetabolite 5-FU is one of the first-line treatment options for gastrointestinal tumors, but its effects on HCC have been poor [22,23]. Yet, 5-FU still

represents the most widely employed chemotherapeutic drug in the management of hepatoma [24].

In the case of paclitaxel, this drug has been very successful in the treatment of tumors, such as ovarian and breast carcinomas [25], but even though in HCC cells such as HuH7 and HepG2, it is able to induce cytotoxicity [26], when tested in patients with unresectable HCC, it did not show very promising results [27].

Cisplatin, on the other hand, has been used as a chemotherapeutic agent in many cancers, especially in testicular cancer and epidermal carcinomas of several organs, for which the treatment is very successful [28]. In the case of HCC, cisplatin administration has been shown to be more effective than other anticancer agents and in combination with drugs such as 5-FU induces additive and synergistic results [29–32].

In this study, we want to improve the characterization of some of the cell death-related events that are induced by three anti-neoplastic agents in HCC cells, attempt to elucidate the mechanisms of cytotoxicity, and uncover some of the reasons that might explain the resistance of HCC to chemotherapy based on the results and the possible differences observed in two different hepatoma cell lines.

2. Materials and methods

2.1. Reagents and antibodies

Dulbecco's essential medium, fetal bovine serum and MTT were obtained from Sigma Chemical Co. (St. Louis, MO). Antibodies against Bcl-xL, Bax, survivin and procaspase-3 were obtained from Santa Cruz Biotechnology (San Diego, CA). Horseradish peroxidase-conjugated anti-mouse whole IgG was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PE). Cisplatin, paclitaxel and 5-FU were donated by Chemotherapy Section of the Hospital Calderón Guardia. Cellular DNA Fragmentation ELISA (Cat. No. 1585045) was obtained from Roche Molecular Biochemicals (Mannheim, Germany).

2.2. Cell culture and treatment

Two different human hepatoma cell lines, HepG2 and Hep3B (obtained from American Type Culture Collection, ATCC) were maintained in Dulbecco's essential medium supplemented with 10% fetal bovine serum, 2 mmol/l glutamine, 100 IU/ml penicillin and amphotericin B in a 37 °C humidified incubator under an atmosphere of 7% CO_2 in air. For the experiments, cells (10^5/well) were plated on 96-well plates and allowed to adhere overnight. Various concentrations of 5-FU, cisplatin and paclitaxel were added to the plates in 100 μl of fresh medium and were incubated for different time periods as indicated in the figures.

2.3. Assessment of cytotoxicity by MTT assay

Cells were treated for 48 h with the drugs and after treatment, 10 μl of MTT reagent (5 mg/ml in PBS) were added to the culture medium. After approximately 1 h of incubation at 37 °C, culture medium was carefully removed and 100 μl of 95% ethanol were added to the wells. Plates were read at 570 nm and results were expressed as viability percentages using the drug solvents as negative controls (100% of viability). Stock solutions of cisplatin, paclitaxel and 5-FU were dissolved in PBS.

2.4. Assessment of apoptosis by the photometric enzyme-linked immunosorbent assay (ELISA) for detection of BrdU-labeled DNA fragments

Briefly, cells were removed from the plates with Trypsin-EDTA solution and labeled for 4–6 h with BrdU at 37 °C. After removing the medium containing BrdU, cells were plated and allowed to adhere overnight. After different treatments, supernatants were removed and kept at −70 °C for no more than days. Cells were lysed with the appropriate solution for 30 min and the extracts were removed and also kept at −70 °C. The ELISA was performed according to the manufacturer's instructions and the results are expressed as absorbencies at 450 nm.

2.5. Western blot analyses

Cells were treated for 48 h with concentrations of 10 μg/ml, 15 μg/ml and 5 mg/ml of cisplatin, paclitaxel and 5-FU, respectively. After treatment, cells were removed from the plates, lysed with buffer (50 mmol/l Tris–HCl, 50 mmol/l NaCl, pH 8.0) and sonicated for 1–2 min on ice. Equal amounts of protein from extracts were separated by 11 or 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. Blots were blocked with 2% dried milk and incubated for 2 h with the primary antibodies at concentrations of 1:200. After washings, blots were incubated with peroxidase-conjugated anti-mouse IgG for two additional hours at concentrations of 1:2000. Blots were developed adding 2 mg/ml 4-chloronaphtol in 0.02 M Tris, pH 7.5 (containing 0.025% H_2O_2). Equal loading was determined using a monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and previous measurement of protein concentration of the samples.

2.6. Statistical analysis

Student's *t*-test analysis of DNA fragmentation was obtained using the InfoStat Statistics Program.

3. Results

3.1. Cytotoxicity induced by chemotherapeutic drugs in hepatoma cells

Both cell lines, Hep3B and HepG2, were highly susceptible to cisplatin and paclitaxel when cytotoxicity was measured by MTT assay after 48 h of treatment (Fig. 1). At concentrations

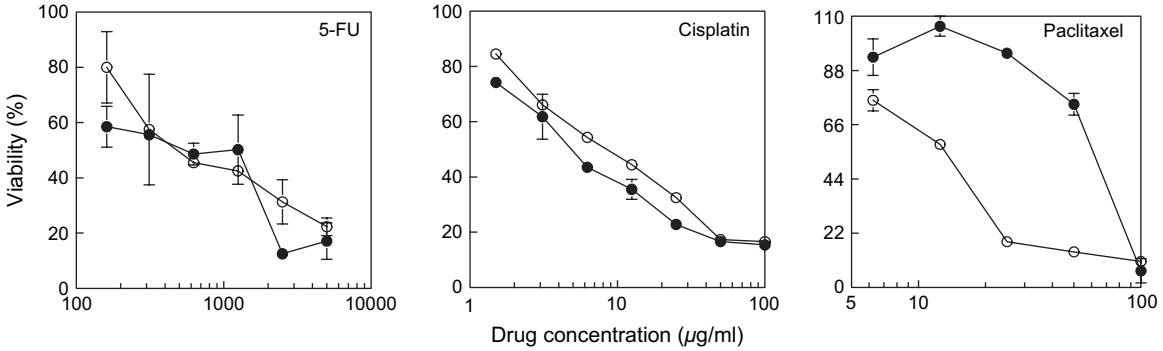

Fig. 1. Cytotoxicity of hepatoma cells induced by chemotherapeutic drugs. After 48 h of treatment with the drugs, MTT activity was read at 570 nm and values were expressed as viability percentage. Filled circles represent Hep3B cells and open circles represent HepG2 cells. The results are presented as a mean + SE of triplicates of one representative experiment performed three times.

of 100 μg/ml (highest dose tested) both drugs were able to induce more than 80% of toxicity in both cell lines. We observed that 5-FU was active at a higher concentration than the other drugs, after 48 h of treatment (Fig. 1). 5-FU and cisplatin did not show significant differences between the two cell lines, whereas treatment with paclitaxel induced a stronger effect in HepG2 cells, at concentrations starting approximately at 10 μg/ml (Fig. 1). The lower concentration of 5-FU tested (about 150 μg/ml) did show a significant difference between both cell lines, showing that Hep3B could indeed be more susceptible to the treatment.

3.2. DNA fragmentation induced by chemotherapeutic drugs in hepatoma cells

DNA fragmentation tests showed that cisplatin (10 μg/ml) and paclitaxel (15 μg/ml) induced some cytotoxic effect in both cell lines after 48 h of treatment. At this time point it was difficult to conclude whether the effect was associated with apoptosis or necrosis since in some cases DNA fragments were observed in supernatants and in other cases in cell extracts (Fig. 2). Yet, cisplatin induced a clear cytotoxic effect (necrosis and apoptosis) in Hep3B cells but not in HepG2 whereas paclitaxel induced significant apoptosis in HepG2 cells but not in Hep3B.

5-FU (5 mg/ml) seemed to induce a significant effect that was associated with necrosis in Hep3B cells, whereas apoptosis and necrosis were observed in HepG2 cells after 48 h of treatment (Fig. 2).

To elucidate the primary cell death mechanism occurring in these hepatoma cells, we determined the DNA fragmentation effect of the drugs every 2 h for the first 8 h of treatment (Fig. 3). 5-FU (5 mg/ml) was able to induce primary necrosis in Hep3B cells, given that DNA fragments were observed in the supernatants from the first hour of treatment. In HepG2, the effect was very low during the early period, and the first statistically significant peak (8 h) was associated with apoptosis (Fig. 3B). Paclitaxel (10 μg/ml), clearly induced apoptosis in both cell lines (Fig. 3) since DNA fragments never significantly appeared in the supernatants up to 8 h of treatment.

Cisplatin seems to induce primary necrosis at a concentration of 100 μg/ml (Fig. 3), but some cells clearly showed signs of apoptosis, which indicates that the effect is probably concentration-dependent.

3.3. Effect of chemotherapeutic drugs in apoptosis-related genes Bcl-xL and Bax

As we can see in Fig. 4, treatment with 5-FU and paclitaxel for 48 h did not induce perceptible changes in the expression of anti-apoptotic protein Bcl-xL in Hep3B cells. On the other hand, 5-FU treatment induced a decrease in protein levels in HepG2 cells. Cisplatin did induce a clear decrease in the expression of this protein in both cell lines.

In the case of pro-apoptotic protein Bax, there was no evident up-regulation observed in cells treated with any of the chemotherapeutic drugs (results not shown).

3.4. Effect of chemotherapeutic drugs in survivin expression and caspase-3 activation

Survivin was up-regulated in Hep3B cells treated with the drugs. HepG2 cells, on the other hand, showed a decrease in protein expression by treatment with 5FU and paclitaxel and no change was observed in the case of cisplatin treatment (Fig. 5).

According to determination of pro-caspase-3 disappearance by Western Blot, Hep3B cells did not show activation of this enzyme after 48 h of treatment with the drugs. In the case of HepG2 cells, we observed a strong decrease in protein levels after treatment with 5-FU and paclitaxel, and a small effect when cisplatin was used (Fig. 6), indicating possible activation of the effector caspase.

4. Discussion

Advances in understanding the molecular mechanisms of cell death in tumor cells have increased the development of several potential approaches for therapeutic intervention. In the case of cancer treatment, it is important to know the mechanisms by which chemotherapeutic drugs induce cell death, so

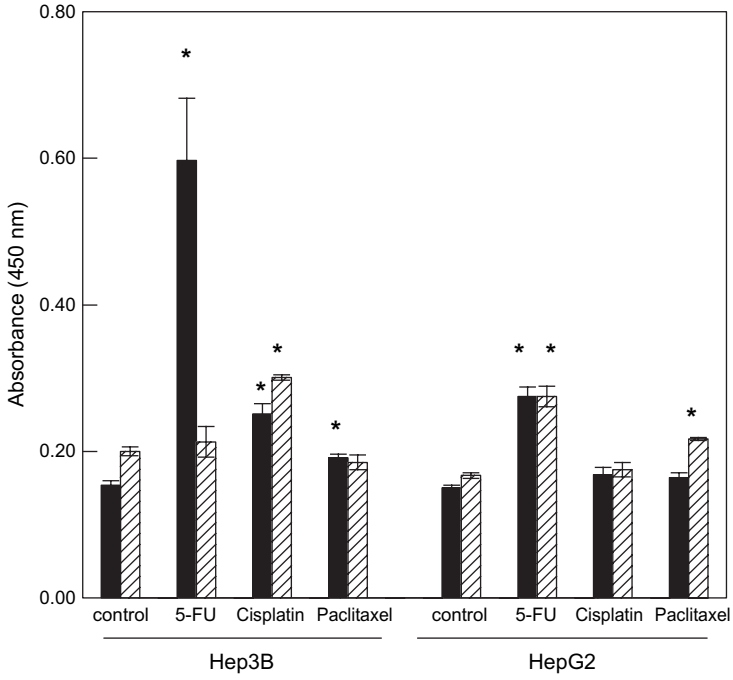

Fig. 2. DNA fragmentation of hepatoma cells induced by chemotherapeutic drugs. After 48 h of treatment supernatants were separated, cells were lysed and DNA fragmentation was measured according to the manufacturer's instructions. Absorbance at 450 nm represents the amount of fragmented DNA observed in the supernatant (black bars) and cell extract (dashed bars). The presence of DNA fragments in the supernatant indicates signs of necrosis due to plasma membrane disruption. Results are expressed as the mean + SE of triplicates of one representative experiment performed three times. *Statistically significant differences compared to the respective control, $p < 0.05$.

that one can select the best treatment for each type of tumor to eliminate the malignant cells and, at the same time, avoid tumor resistance to the treatment.

Several agents have been used for the treatment of HCC, alone or in combination [33]. Among them are the ones tested in this study, with 5-FU being the most commonly used agent [24].

Statistically significant differences between Hep3B and HepG2 cell lines were not observed for 5-FU and cisplatin treatments, suggesting that cytotoxic mechanisms were p53-independent or mediated by two different pathways in these cell lines. In contrast, when cells were treated with paclitaxel, statistically significant differences were observed, HepG2 cells were more sensitive than Hep3B, suggesting a p53-dependent mechanism.

Since measuring MTT activity does not give us clear information about the type of death cells were suffering, we quantified DNA fragments present in cell extracts and supernatants, to discriminate between apoptosis and necrosis. At 48 h, it was observed that 5-FU had a more pronounced necrotic effect in Hep3B cells, whereas HepG2 cells showed both significant necrosis and apoptosis. This suggests that high doses of 5-FU could be inducing different types of cell death in these two cell lines, which is reasonable taking into account that both cells differ in the expression of several apoptosis-related genes [12,13]. However, another possibility that must be taken into account is that while apoptosis was the first process triggered, at the time of the assay cells might have already been necrotic due to a secondary mechanism. To try to elucidate which cell death mechanism came first, we measured DNA fragmentation at earlier points and by a time course that would show, at the same time, the appearance of fragments in the supernatants and cell extracts. We observed that 5-FU was able to disrupt Hep3B cell membranes from the first 2 h of treatment, showing that necrosis was probably the first mechanism triggered at the drug concentrations tested here. However, even though the effect was very clear in the case of Hep3B cells, HepG2 cells have very low levels of DNA fragmentation at these time points, which makes the results more difficult to interpret. In fact, the first statistically significant point was observed in the cell extract and not in the supernatant, suggesting that, in the case of HepG2, the primary mechanism of death is probably associated with apoptosis.

It has been shown that paclitaxel can induce apoptosis in HepG2 cells by a mechanism associated with p53 and down-regulation of Bcl-xL [12,13]. Nonetheless, at the concentration tested here, paclitaxel did not induce a decrease in Bcl-xL protein levels in any of the cell lines, and did not affect Bax expression. Regarding the expression of Bcl-2 family pro-apoptotic proteins, it has been previously demonstrated that several HCC cell lines exposed to paclitaxel do not suffer any changes in the expression of Bax or Bad [34]. The significantly higher cytotoxic effect induced by paclitaxel in HepG2 cells compared to Hep3B, strongly suggests that the apoptotic mechanism may also be mediated by p53.

Contrary to what we observed with paclitaxel, Bcl-xL was down-regulated when both cell lines were treated with cisplatin and when HepG2 cells were exposed to 5-FU.

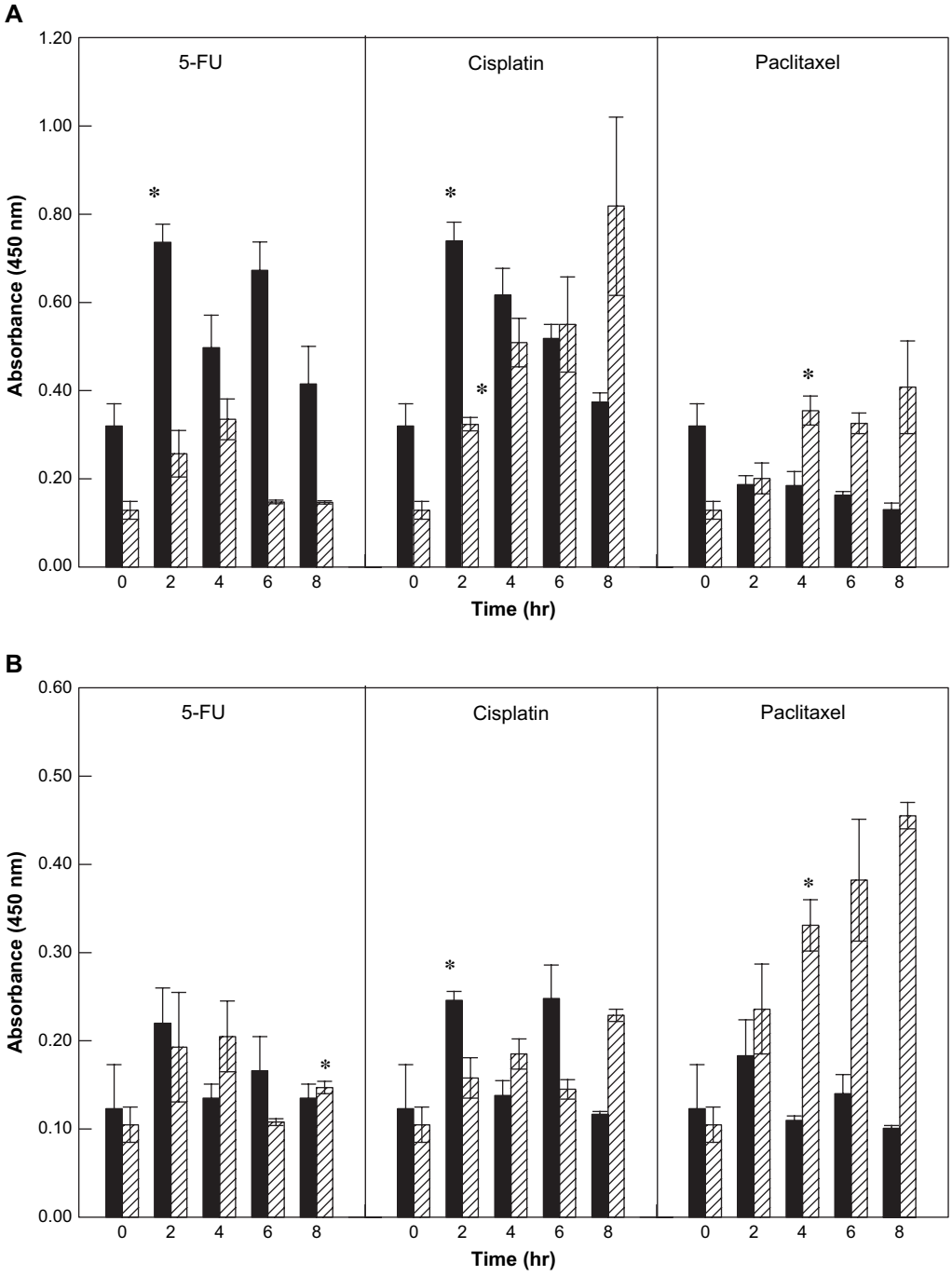

Fig. 3. Early DNA fragmentation time course of hepatoma cells treated with chemotherapeutic drugs. DNA fragments were quantified in the supernatants (black bars) and cell extracts (dashed bars) after different periods of treatment according to the manufacturer's instructions. (A) Hep3B cells; (B) HepG2 cells. Results are expressed as mean + SE of triplicates of one representative experiment performed twice. *First time points that present statistically significant differences when compared to the respective control, $p < 0.05$.

Cisplatin has been shown to induce apoptosis in human hepatoma cell lines, but the mechanism is not fully understood. Kim and collaborators [35] recently showed that cisplatin-induced apoptosis in Hep3B cells is associated with mitochondrial dysregulation. They observed loss of mitochondrial membrane potential, an efflux of cytochrome *c* and Smac/Diablo as well as activation of caspases-3, -8 and -9 in cells exposed to the drug. In our study, however, we were not able to observe caspase-3 activation when both hepatoma cell lines were exposed to cisplatin. This result is consistent with some reports that show that cisplatin-induced apoptosis can be mediated through caspase-independent pathways [36,37], which leads to the possibility that other proteases could be involved in the process. Besides, as we observed here for Hep3B cells, some studies have reported that cisplatin can induce either necrosis or apoptosis, since its mechanism of

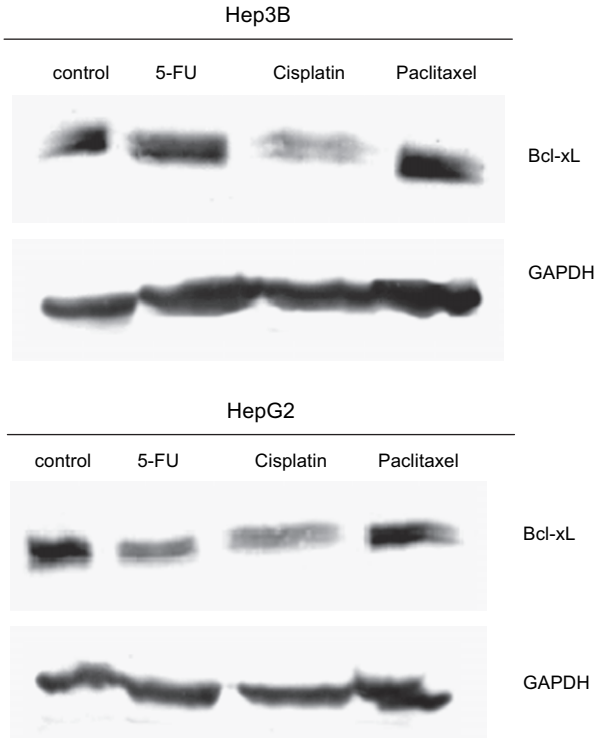

Fig. 4. Expression of Bcl-xL in hepatoma cells after 48 h of treatment with chemotherapeutic drugs. Equal loading was assessed by measuring protein concentration of the samples and by using an antibody against GAPDH. Results show one of three representative experiments.

toxicity is concentration-dependent [28]. When we determined DNA fragmentation in cells treated with 100 μg/ml of cisplatin at earlier times, we detected that necrosis was probably the primary effect induced, because membranes were being disrupted from the first 2 h (Fig. 3).

In our study, pro-apoptotic protein Bax did not show changes in its expression with any of the treatments. Studies using cisplatin have shown that Bax expression does not change, but the protein re-localizes on the mitochondria, where is expected to trigger the release of several apoptotic factors [35]. This event remains to be clarified in hepatoma cell lines treated with this and other chemotherapeutic drugs.

Zhu et al. demonstrated that the presence of Bcl-xL in human colorectal cancers provides some degree of resistance against 5-FU-induced apoptosis [38]. We observed here that Hep3B cells, which did not change Bcl-xL levels with the treatment, did not show apoptosis but necrosis, whereas HepG2 cells suffered apoptosis (and possibly secondary necrosis) at the same time as the levels of this anti-apoptotic protein were decreased.

A recent study has shown that 5-FU can activate several caspases in Hep3B cells by a two-stage mechanism [39]. They showed that caspases were fully activated at 72 h, whereas apoptosis was detected earlier (at 24 h). They suggested a first stage that takes place with weakly activated caspases and a second stage where Bcl-2 family member Bid takes control and further activates several caspases, increasing apoptosis. According to this study, and since we measured

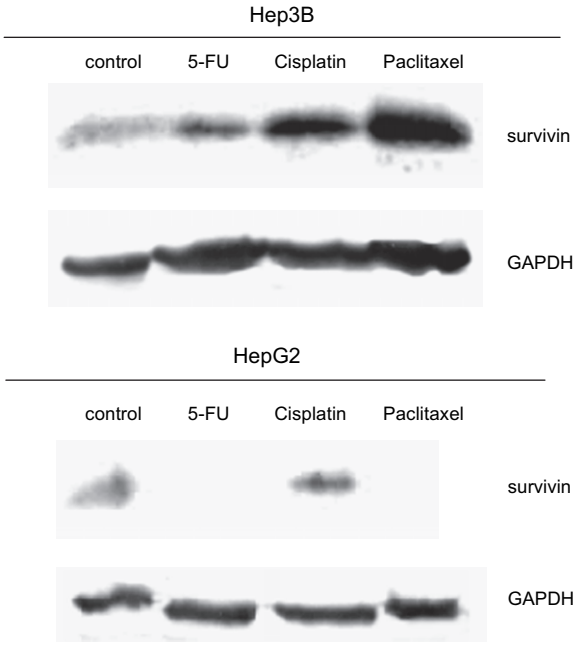

Fig. 5. Expression of survivin after 48 h of treatment with chemotherapeutic drugs. Equal loading was assessed by measuring protein concentration of the samples and by using an antibody against GAPDH. Results show one of three representative experiments.

caspase-3 activation at 48 h, we probably did not detect any activation in Hep3B cells treated with 5-FU, because they were still in stage one. Also since DNA fragmentation ELISA shows cells are suffering mainly necrosis, it was not necessarily expected that caspases would be activated.

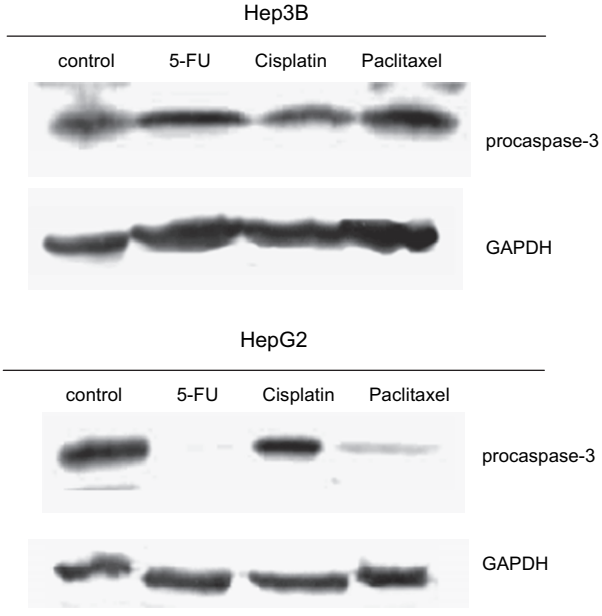

Fig. 6. Activation of caspase-3 after 48 h of treatment with chemotherapeutic drugs. Antibodies recognize unprocessed caspase-3. Equal loading was assessed by measuring the protein concentration of the samples and by using an antibody against GAPDH. Results show one of three representative experiments.

HepG2 cells did show a decrease in procaspase-3 after treatment with 5-FU suggesting a different mechanism of cell death is being triggered in each hepatoma cell line, which could also explain the differences seen in DNA fragmentation ELISA between both cell types (see above). This might also mean that the presence of wt-p53 is relevant for caspase-3 activation, but not essential for cell death induction.

In addition, we saw that HepG2 cells did show a decrease in procaspase-3 after treatment with paclitaxel, suggesting a caspase-3-dependent apoptotic pathway, as has been show in HL-60 human leukemia cell line [40], however Hep3B cells did not present changes in caspase-3 activity, suggesting different mechanisms of cell death triggered by this drug in both hepatoma cell lines.

Some studies have shown that IAP survivin expression is inversely associated with apoptosis in HCC [20]. We showed here that survivin increases its expression in Hep3B cells treated with cisplatin, paclitaxel and 5-FU. This increase in survivin could explain the lack of caspase-3 activation in this cell line, since caspase-3 can be activated by caspase-9, which has been shown to be the target of inhibition by survivin [41]. Something completely different was observed in HepG2 cells, where we determined survivin down-regulation when cells were treated with 5-FU and paclitaxel.

We think it is likely that survivin could be, at least in part, responsible for the resistance of Hep3B cells to some chemotherapeutic drugs such as 5-FU and probably paclitaxel. When survivin is silenced with antisense oligonucleotides, an increase in apoptotic index is observed in HepG2 cells [21], which indicates that the apoptosis threshold decreases when survivin is down-regulated. This could explain the observations made when HepG2 cells are treated with 5-FU and paclitaxel. These two treatments induce apoptosis at the same time as survivin is being down-regulated. It is important to point out that Bcl-xL was also down-regulated by 5-FU in HepG2 cells, which could indicate that in this cell line, apoptosis occurs through a combined effect in the decline of these two very important anti-apoptotic proteins. Moreover, with the decreased expression of these two inhibitors (Bcl-xL and survivin), apoptosis could take place by activation of several caspases such as caspase-3. A similar effect, where the combination of these two inhibitors of apoptosis was reduced simultaneously in Hep3B cells, was observed with natural retinoid ATRA. Our group demonstrated that treatment with this agent clearly resulted in apoptosis [18].

The other possibility could be that an increase in the expression of survivin is able to switch between the different types of cell death. In other words, instead of apoptosis, a cell with high survivin expression could suffer necrosis or even other types of cell death such as autophagy [42]. This could apply in the case where cells are treated with cisplatin, which, in this study, induces cell death by early membrane disruption and no caspase-3 activation.

The higher resistance to paclitaxel shown by Hep3B cells compared to HepG2, could explain the lack of improvement of patients with unresectable HCC who were treated with this drug, as was observed in clinical trials [27]. Cisplatin and 5-FU, on the other hand, have a low but significant effect in HCC patients [29,30] and 5-FU remains the first choice in the chemotherapeutic treatment of these patients.

Overall, we observed that cisplatin and 5-FU have the basis for being promising agents for the treatment of HCC, especially cisplatin, whereas paclitaxel does not seem to be a good option, at least in p53-negative cells. Moreover, we have to emphasize that resistance to therapy due to, among other things, high expression of survivin, is an important problem and despite strategies having been suggested to solve this problem [43], more experimental studies are needed.

The low response that HCC patients have to chemotherapy indicates that new drugs and new drug combinations have to be developed and tested on hepatoma cells, in order to find better approaches for the treatment of this drug-resistant type of cancer. We and other groups have been working on the use of natural and synthetic retinoids for the treatment of HCC [17,18,23]. Even though retinoids have been extensively used for the treatment of several diseases, including some types of cancer [44] and clinical studies have been done with synthetic retinoids [45,46], more studies are necessary to confirm their efficacy for the treatment of HCC. Combinations of drugs have been more successful than one-agent protocols and combined therapies including 5-FU and cisplatin together with retinoids need to be tested in the future.

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