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ORIGINAL ARTICLE

Terence Kin-Wah Lee · Tracy Ching-Man Lau
Irene Oi-Lin Ng

Doxorubicin-induced apoptosis and chemosensitivity in hepatoma cell lines

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Abstract *Purpose*: Doxorubicin (DOX) is a commonly used anticancer drug which causes DNA damage and kills cancer cells mainly by apoptosis. However, the process leading to killing of cancer cells and the molecular basis of resistance to DOX are not well understood. To evaluate the role of *p53* and the cellular effects of DOX on hepatoma cell lines, we examined three hepatoma cell lines with different *p53* status – Huh-7 (mutated *p53*), HepG2 (wild-type *p53*) and Hep3B (deleted *p53*). *Methods*: The chemosensitivity of the three hepatoma cell lines was assessed using the MTT assay, and cell cycle distribution was evaluated by flow cytometry. Western blotting and immunostaining were employed to examine the protein alterations in response to DOX treatment, and a DNA fragmentation assay was performed to detect apoptosis. *Results*: Of the three cell lines, HepG2 was found to be most resistant to DOX, followed by Hep3B, and Huh-7 was most sensitive to DOX treatment. HepG2 showed G_1 arrest 24 h after drug administration and upregulation of p53 protein level in a time-dependent manner. In Hep3B cells (deleted *p53*), G_2/M phase arrest was observed soon after drug administration, accompanied by induced apoptosis that was *p53*-independent. In Huh-7 cells (mutated *p53*), which were most sensitive to DOX, there was neither G_1 nor G_2 arrest, and the level of p53 mutated protein was downregulated after DOX treatment. MDM2 and p27 proteins were downregulated in all cell lines independently of *p53* status. p21 was upregulated following p53 activation at low doses of DOX in HepG2 cells, but at higher doses, p21 was downregulated in Huh-7 and HepG2 cells. *Conclusion*: DOX confers different chemosensitivity on different hepatoma cell lines with different *p53* status. The contrasting relationships between chemosensitivity and *p53* status and expression suggest that DOX-induced apoptosis and cell death involve pathways that are independent of *p53*.

Keywords Apoptosis · Chemosensitivity · DOX · p53 p21

Introduction

Hepatocellular carcinoma (HCC) is one of the commonest malignancies worldwide [38, 41]. Most patients present at an advanced stage when operation is no longer feasible. Chemotherapy is a common treatment modality for inoperable HCC. Among the various chemotherapeutic drugs, doxorubicin (DOX) is widely used for HCC. DOX is a cytotoxic anthracycline antibiotic isolated from culture of *Streptomyces peuxetus* [36]. The underlying mechanism is related to its ability to bind to DNA and inhibit nucleic acid synthesis. DOX binds to DNA by intercalation and this results in protein-concealed DNA strand breaks as a result of DNA topoisomerase II poisoning [17]. Cell culture studies have demonstrated rapid cell penetration and perinucleolar chromatin binding, rapid inhibition of mitotic activity and nucleic acid synthesis, mutagenesis and chromosomal aberrations. Although the molecular targets of this anticancer drug have been identified, the process leading to killing of the cancer cells, particularly in relation to cell cycle regulation and apoptosis, is unclear.

The human *p53* tumor-suppressor gene encodes a 393 amino acid nuclear phosphoprotein which is involved in the regulation of cell proliferation [28]. Mutations of the

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T.K.-W. Lee · T.C.-M. Lau · I.O.-L. Ng (✉)
Department of Pathology and Center for the Study of Liver Disease, University of Hong Kong, Hong Kong, China
E-mail: iolng@hku.hk
Tel.: +852-28554859
Fax: +852-28725197

I.O.-L. Ng
Department of Pathology, University of Hong Kong, Queen Mary Hospital, Pokfulam Road, Pokfulam, Hong Kong, China

p53 gene have been observed with a high prevalence in more than half of human malignancies derived from epithelial, mesenchymal, hematopoietic, and lymphoid tissues [16]. The most striking *p53* mutational spectrum found in human cancers is that of HCC, particularly in southeast Asia where hepatitis B virus infection and aflatoxin B1 exposure are the major risk factors [3]. The *p53* gene product has been implicated as a molecule of central importance [40]. It has a role in apoptosis because of its involvement in DNA damage-induced G_1 arrest, apoptosis [8, 23, 24] and DNA repair [2]. The p53 protein senses genotoxic stress or DNA damage [22, 26] and this results in nuclear accumulation of p53 following exposure to DNA-damaging agents such as cisplatin, DOX and cyclophosphamide [10]. Subsequently, p53 activates the transcription of several genes whose products are involved in either DNA repair or apoptosis. These genes include *p21*, *MDM2* and *cyclin G* [20, 21]. Loss of wild-type *p53* function may enhance cellular resistance to a variety of chemotherapeutic drugs [11, 24]. The vulnerability to a variety of chemotherapeutic agents can be greatly reduced by mutations that abolish *p53*-dependent apoptosis, but this has not been observed in all cases [39]. The role of wild-type *p53* gene in chemosensitivity remains controversial, possibly because of the differences in the cell types studied and in their susceptibility to apoptosis.

In this study, we examined the possible role of the *p53* gene in chemosensitivity, cell cycle changes, and apoptosis following exposure to DOX in three different hepatoma cell lines with different p53 status: Huh-7 (mutated *p53*), Hep3B (deleted *p53*) and HepG2 (wild-type *p53*). DNA damage-related proteins including p53, p21, MDM2 and cell cycle-related protein p27 were also examined.

Materials and methods

Cell lines

The human HCC cell line Huh-7 [34] (a gift from Dr. H. Nakabayashi, Hokkaido University School of Medicine) and the human hepatoblastoma cell line HepG2 (American type Culture Collection, HB-8065) were maintained in Dulbecco's modified Eagle's minimal essential medium with high glucose (Gibco BRL, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, Mo.), 50 U/ml penicillin G, and 50 μg/ml streptomycin (Gibco BRL) at 37 °C in a humidified atmosphere containing 5% CO_2. The human HCC cell line Hep3B (American type Culture Collection, HB-8064) was grown in minimal essential medium (Gibco BRL).

Drug and dosage

DOX was obtained from Calbiochem (La Jolla, Calif.). The IC_{50} (see below) was used after dilution in phosphate-buffered saline (PBS) for each experiment in Huh-7, Hep3B and HepG2 cell lines, unless stated otherwise, as in Western blotting experiments.

MTT assay

The MTT assay is a colorimetric assay based on the ability of viable cells to reduce a soluble yellow tetrazolium salt (MTT) to blue formazan crystals. The hepatoma cells were seeded into 96-well plates, and appropriate concentrations of DOX ranging from 0.078 to 40 μg/ml were then added. After 24–72 h, MTT dye, at a concentration at 5 mg/ml (Sigma, St Louis, Mo.), was added and the plates were incubated for 12 h in a moist chamber at 37 °C. Optical density was determined by eluting the dye with dimethyl sulfoxide (Sigma), and the absorbance was measured at 560 nm. At least three independent experiments were performed.

Determination of IC_{10}, IC_{50}, and IC_{90}

To determine the cytotoxic effects of DOX on the hepatoma cell lines, the MTT assay was performed. The MTT assay allowed the determination of IC_{10}, IC_{50}, and IC_{90} from the dose response curves using multiple doses of DOX. IC_{10}, IC_{50}, and IC_{90} were defined as the doses of drug resulting in 10%, 50%, and 90% loss of cell viability, respectively, relative to untreated cells 36 h after DOX treatment.

Cell cycle analysis

After DOX treatment, the DNA content and cell cycle distribution of hepatoma cells grown in six-well plates were determined by flow cytometry. The cells were plated at a low density (5×10^4/well) and were harvested at 0, 3, 6, 12, 24 and 36 h. They were washed twice with PBS and resuspended in 200 μl PBS, followed by the addition of 2 ml 70% ice-cooled ethanol for 30 min at 4 °C. The cells were centrifuged again and the pellets were resuspended in 200 μl PBS. The cell suspensions were kept at 4 °C overnight. RNAse (100 μl, 1 mg/ml) and propidium iodide (100 μl, 100 mg/ml) were added to the cell suspensions followed by incubation at 37 °C for 30 min. An Epics XL-MCL flow cytometer (Beckman Coulter, Calif.) was used with an Epics XL-MCL workstation, version 1.5, for cell cycle evaluation.

DNA fragmentation analysis

Cells were harvested 36 h after DOX treatment, suspended and transferred to tubes containing 10 ml ice-cold ethanol. The fixed cell pellets were resuspended in 40 μl PC buffer (192 parts 0.2 *M* Na_2HPO_4 and 8 parts 0.1 *M* citric acid at pH 7.8) and incubated for 30 min at room temperature. After pelleting at 1000 *g* for 5 min, the supernatant was transferred to another tube and concentrated using a SpeedVac for 15 min at low speed. Nonidet NP40 solution (3 μl, 0.25%) and RNase A solution (1 mg/ml) were added to the concentrated supernatant followed by incubation for 30 min at 37 °C. The mixture was further incubated for 30 min at 37 °C after the addition of 3 μl 1 mg/ml proteinase K solution. DNA fragmentation was analyzed in 1% agarose gel in 1× TBE. The gel was run at 80 V for 3 h and then analyzed using a UV illuminator.

Immunostaining of p53 protein

After DOX treatment, the hepatoma cells were fixed at 0, 3, 6, 12, 24 and 36 h with a 1:1 (v/v) mixture of acetone and methanol, air dried, and washed. Mouse monoclonal antibody against p53 (Santa Cruz Biotechnology, Santa Cruz, Calif.) at a dilution of 1:100 was added and the cells were incubated overnight at 4 °C. The slides were then incubated with rabbit anti-mouse IgG-biotinylated (DAKO, Glostrup, Denmark) at a dilution of 1:100 for 30 min at room temperature. The developed sections were counterstained with Mayer's hematoxylin.

Western blot analysis of protein expression

The levels of p53, MDM2, p27 and p21 were analyzed by Western blotting. The hepatoma cells were lysed and protein extraction was performed after the cells had been harvested 36 h after DOX

treatment at IC_{10}, IC_{50}, and IC_{90}. The samples were separated in 10% SDS-polyacrylamide gel (PAGE) and electrophoretically transferred to PVDF membrane (Amersham, Little Chalfont, UK) using the Bio-Rad electrotransfer system (Bio-Rad Laboratories, Munich, Germany). The membranes were blotted with 10% skimmed milk, washed with TBST (20 m*M* Tris-HCl, 137 m*M* NaCl, 0.1% Tween 20) and then probed with mouse monoclonal antibody against p53 and β-actin (Santa Cruz Biotechnology, Santa Cruz, Calif.), mouse monoclonal antibody against p27 (Transduction Laboratories, Lexington, Ky.), mouse monoclonal antibody against MDM2 (Calbiochem, La Jolla, Calif.), and mouse monoclonal antibody against p21 (Pharmingen, San Diego, Calif.). After washing in TBS containing 0.1% Tween 20, the membrane was incubated with horseradish peroxidase-conjugated rabbit anti-mouse antibody or goat anti-rabbit antibody (Amersham, Little Chalfont, UK) and then visualized by enhanced chemiluminescence according to the manufacturer's recommendations (Amersham).

Results

Determination of IC_{50} and sensitivity to DOX

To determine the cytotoxic effect of DOX on the hepatoma cell lines with different *p53* status, the MTT assay was performed to determine the IC_{10}, IC_{50}, IC_{90} at least three times. As shown in Table 1, the IC_{50} values of Huh-7, Hep3B and HepG2 were 0.47 ± 0.9, 7.1 ± 1.1 and 16.2 ± 0.8 μg/ml, respectively. HepG2 was the most resistant to DOX as compared with Hep3B and Huh-7. The resistance to DOX was approximately twice that of Hep3B and 30 times that of Huh-7 according to the IC_{50} values. Huh-7 was the most sensitive to DOX. The dose response curves are shown in Fig. 1.

Immunostaining for p53 protein

As p53 protein accumulation is often observed in response to DNA damage, suggesting a possible causal relationship between DNA damage and elevation of p53 levels, we evaluated these hepatoma cells for the presence of nuclear p53 protein. In HepG2 cells, a weak basal level of p53 nuclear staining was observed. There was a clear nuclear accumulation of p53 protein 12 h after DOX treatment but no significant further increase in p53 protein level beyond that time-point (Fig. 2A). In untreated Huh-7 cells, there was strong p53 nuclear expression, likely due to the prolonged half-life of the mutated p53 protein. The level of p53 nuclear protein started to decrease 24 h after treatment (Fig. 2B). In Hep3B cells, there was no immunostaining for p53 protein either before or after drug treatment because of the deleted *p53* (Fig. 2C).

Table 1 Determination of the cytotoxic effects of DOX on Huh-7, HepG2 and Hep3B cells. IC_{10}, IC_{50} and IC_{90} are the concentrations of DOX giving 10%, 50% and 90% growth inhibition, respectively, in the MTT assay at 36 h after DOX treatment. The inhibitory concentrations of DOX were calculated by linear interpolation between the values immediately above and below the percentage inhibition in question. Values are the means ± SD in micrograms per microliter from at least three independent experiments

Cell line	p53 status	IC_{10}	IC_{50}	IC_{90}
Huh-7	Mutated at codon 220	0.1 ± 3.8	0.47 ± 0.9	7.8 ± 2.1
Hep3B	Deleted	1.0 ± 3.3	7.1 ± 1.1	18 ± 4.2
HepG2	Wild-type	3.8 ± 1.2	16.2 ± 0.8	33 ± 4.2

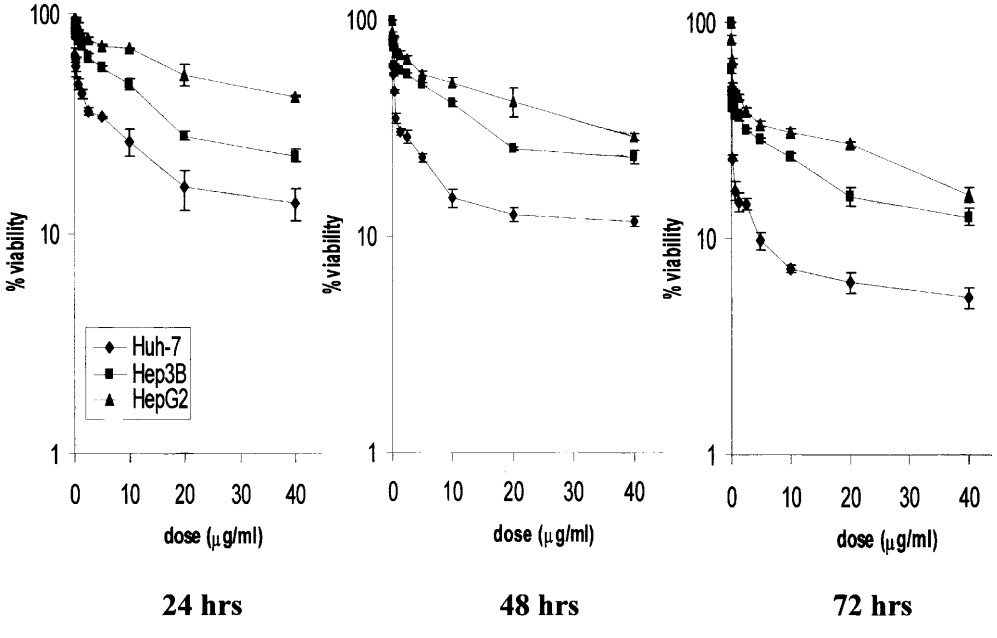

Fig. 1 Chemosensitivity of HepG2, Hep3B and Huh-7 towards DOX treatment. To evaluate the drug sensitivity, the MTT assay was performed in cells 24, 48 and 72 h after DOX treatment respectively. HepG2 was most resistant, and Huh-7 was least resistant to DOX

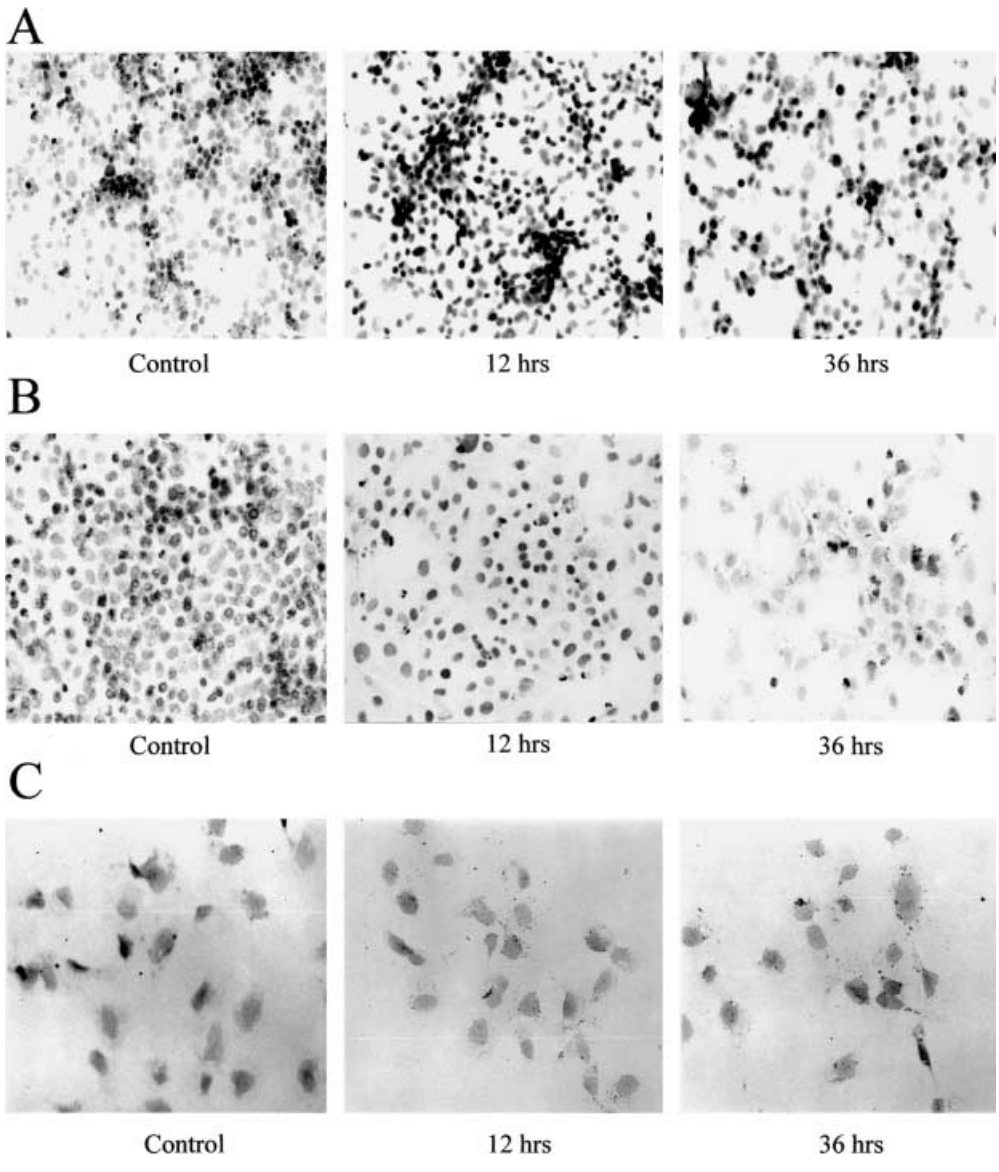

Fig. 2A–C Immunocytochemical localization of p53 with and without DOX treatment in (**A**) HepG2, (**B**) Huh-7 and (**C**) Hep3B cells. The cells were harvested before (*control*) and 12 and 36 h after DOX treatment. There was nuclear accumulation of p53 protein in HepG2 cells while a decrease in nuclear accumulation of p53 was observed in Huh-7 cells. No p53 was detected in Hep3B cells

Analysis of cell cycle changes

DNA damage-induced G_1 block in the cell cycle depends on a functionally intact *p53* gene. To evaluate the possible effects on the cell cycle of the different p53 status, cell cycle changes were analyzed by flow cytometry in Huh-7, HepG2 and Hep3B cells at 3, 6, 12, 24 and 36 h after DOX treatment. In HepG2 cells, which were most resistant to DOX among the three hepatoma cell lines, there was no significant change in the cell cycle 3 h after DOX treatment. However, a significant G_1 arrest was observed at 12 h and a sub-G_1 peak at 36 h (Fig. 3A), the latter indicating apoptosis. In Hep3B cells which were moderately sensitive to DOX, there was a significant G_2 arrest 3 h after DOX treatment, and an apoptotic peak was observed at 12 h (Fig. 3B). In Huh-7 cells, which was most sensitive to DOX among the three cell lines, there was no significant G_1 or G_2 arrest after DOX treatment, and an apoptotic peak was observed 12 h after treatment and thereafter (Fig. 3C).

DNA fragmentation analysis

Apoptosis induced by DOX treatment was analyzed by DNA laddering. DNA laddering was observed 36 h after DOX treatment in all three hepatoma cell lines (Fig. 4).

Analysis of DNA damage-induced and cell cycle-related proteins by Western blotting

The p53, MDM2, p27 and p21 proteins were evaluated by Western blotting in HepG2, Huh-7 and Hep3B cells after treatment with DOX at IC_{10}, IC_{50}, and IC_{90}

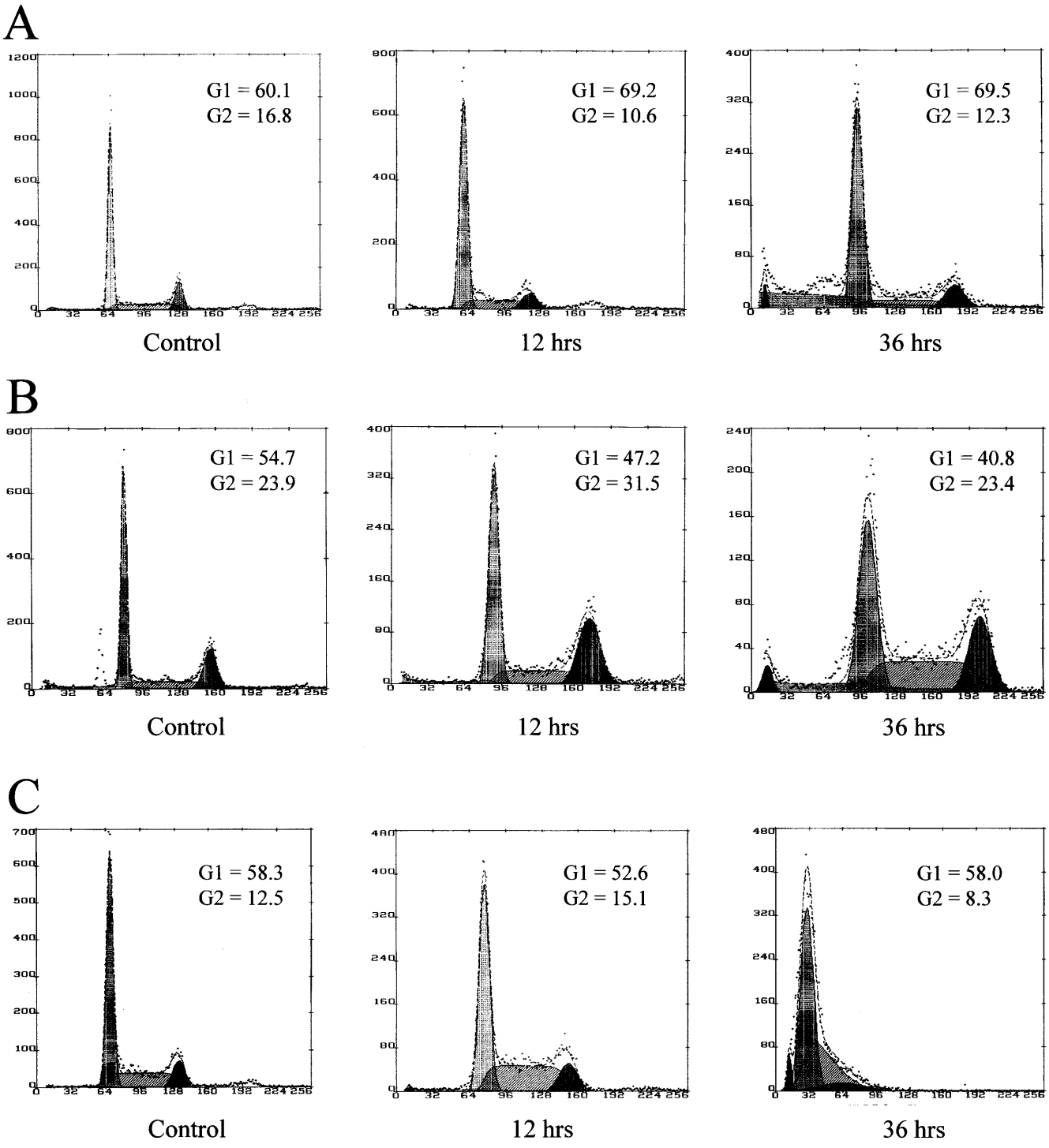

Fig. 3A–C Flow cytometric analysis of (**A**) HepG2, (**B**) Huh-7 and (**C**) Hep3B cells following DOX treatment. The cells were harvested before (*control*) and 3, 6, 12, 24 and 36 h after DOX treatment

(Fig. 5). In HepG2 cells, with DOX treatment at IC_{10}, p21 was upregulated following p53 activation at 12 h accompanied by downregulation of MDM2 at 24 h and thereafter. The p27 protein level was increased at 12 h and thereafter. With DOX at IC_{50}, both p21 and MDM2 protein levels were reduced, whereas p53 protein was increased significantly at 6 h peaking at 24 h. There was no further increase in the p53 protein level. The p27 protein level had decreased significantly by 3 h after DOX treatment, but increased again until 12 h and then again decreased. At IC_{90}, p21 and MDM2 were downregulated in a time-dependent manner, whereas p53 protein was upregulated. Time-dependent downregulation of p27 was also observed. In Huh-7 cells, the MDM2, p27 and p21 protein levels decreased progressively after DOX treatment in a dose-dependent manner.

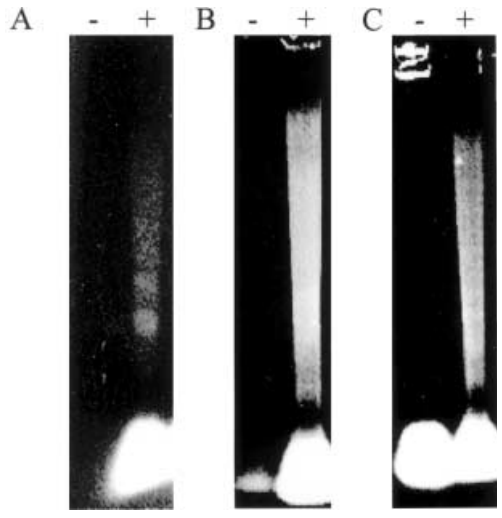

Fig. 4A–C Induction of apoptosis in HepG2, Hep3B and Huh-7 cells by DOX treatment for 36 h. Internucleosomal DNA fragmentation (DNA laddering) was observed 36 h after DOX treatment in (**A**) HepG2, (**B**) Hep3B and (**C**) Huh-7 cells

The p53 levels had dropped significantly by 24 h after treatment and later at both IC_{50} and IC_{90}. In Hep3B cells, there were no detectable p53 and p21 proteins due to the deleted *p53*. MDM2 and p27 proteins decreased progressively in a dose- and time-dependent manner.

Discussion

In this study, we examined the chemosensitivity of three hepatoma cell lines towards DOX, which is widely employed in the treatment of HCC. We found that HepG2 with wild-type *p53* was most resistant to DOX compared with Huh7 with mutated *p53* and Hep3B with deleted *p53*. This result was consistent with those found in other non-hepatoma cell lines with wild-type *p53* [25]. Among HCC cell lines, however, Huh-7 has been reported to be significantly more resistant than HepG2 to bleomycin [32]. These findings support the notion that chemoresistance may be tissue-specific and the role of *p53* in drug sensitivity may be dependent on the class of the chemotherapeutic agent studied [9, 14, 43, 46].

To determine the effects of DOX on the cell cycle, flow cytometric analysis was employed. In HepG2 cells, G_1 arrest was induced and a sub-G_1 peak became obvious 12 h after treatment. The appearance of the G_1 arrest paralleled p53 nuclear accumulation. These results are consistent with a previous finding that the p53 gene product is required to arrest cells in the G_1 phase following DNA damage, at least that caused by ionizing radiation [19]. This arrest is believed to allow DNA repair to maintain chromosomal fidelity for survival [12]. In Hep3B cells, G_2 arrest occurred soon after DOX treatment, and significant apoptosis was observed subsequently. In Hep3B cells, DOX induced G_2/M arrest followed by *p53*-independent apoptosis. Other than wild-type *p53*, other mechanisms or pathways may account for the G_2/M arrest [43]. In Huh-7 cells, there was neither G_1 nor G_2 arrest observed after DOX treatment. The results suggested that the absence of G_1 and G_2 arrest might result in increased chemosensitivity to DOX due to direct execution of apoptosis. After treatment with DNA-damaging agents, the loss of checkpoint function in *p53*-mutant cells (Huh-7) resulted in an increased sensitivity towards DOX. In contrast, wild-type *p53* in HepG2 was able to arrest damaged cells in G_1, so allowing time for DNA repair and hence decreasing the sensitivity.

Immunostaining and Western blotting both showed a dose-dependent increase in p53 levels and p53 nuclear accumulation in HepG2 after DOX treatment. It is known that agents such as UV, hydrogen peroxide and actinomycin are able to elevate the p53 protein levels leading to p53 accumulation [15, 20, 27]. DNA-damaging agents cause an increase in p53 protein levels in a variety of normal and malignant cells with wild-type *p53* which appears to play a significant role in normal G_1 and G_2 checkpoints [13]. It seems that an inability to effectively repair damaged DNA may result in the generation of potentially lethal genetic abnormalities and cell death. Indeed, transfection of mutated *p53* into cisplatin-resistant human ovarian cancer cells, which originally have high levels of wild-type p53 protein, may change the cells to become significantly sensitive to cisplatin [4]. In Huh-7 cells, there was a strong nuclear accumulation of p53 protein in untreated cells due to the prolonged half-life of the mutated p53 protein.

We observed for the first time a decrease in p53 nuclear accumulation in Huh-7 cells 24 h after DOX treatment at IC_{50}, and the decrease appeared even earlier at 12 h after DOX treatment at IC_{90}. This is in contrast to either an increase or no change in p53 levels by a translational mechanism in response to anticancer drugs in gastric and esophageal cancer cells, which originally express mutant p53 levels [33]. The mechanism for such a decrease in p53 protein levels is not certain but might involve modification at the transcriptional, translational or post-translational levels. In untreated Hep3B cells, since the p53 gene is deleted no detectable p53 protein was observed and, as expected, there was no change in p53 protein level after DOX treatment [29].

With Western blotting, the effects of DOX on various DNA damage and cell cycle-related proteins were determined. Downregulation of both MDM2 and p53 upon DOX treatment was observed in all three cell lines in a dose-dependent and time-dependent manner. MDM2 is thought to be transcriptionally induced by p53 after its stabilization by DNA damage [45]. However, MDM2 can bind to p53 blocking the ability of p53 to function as a transcription factor and tumor suppressor [5, 6, 7, 30, 35, 37]. MDM2 and p53 therefore form an autoregulatory feedback loop in which p53 limits its own activity through the induction of MDM2 [18, 31]. It has been reported that p53 and MDM2 proteins are activated in response to DNA damage due

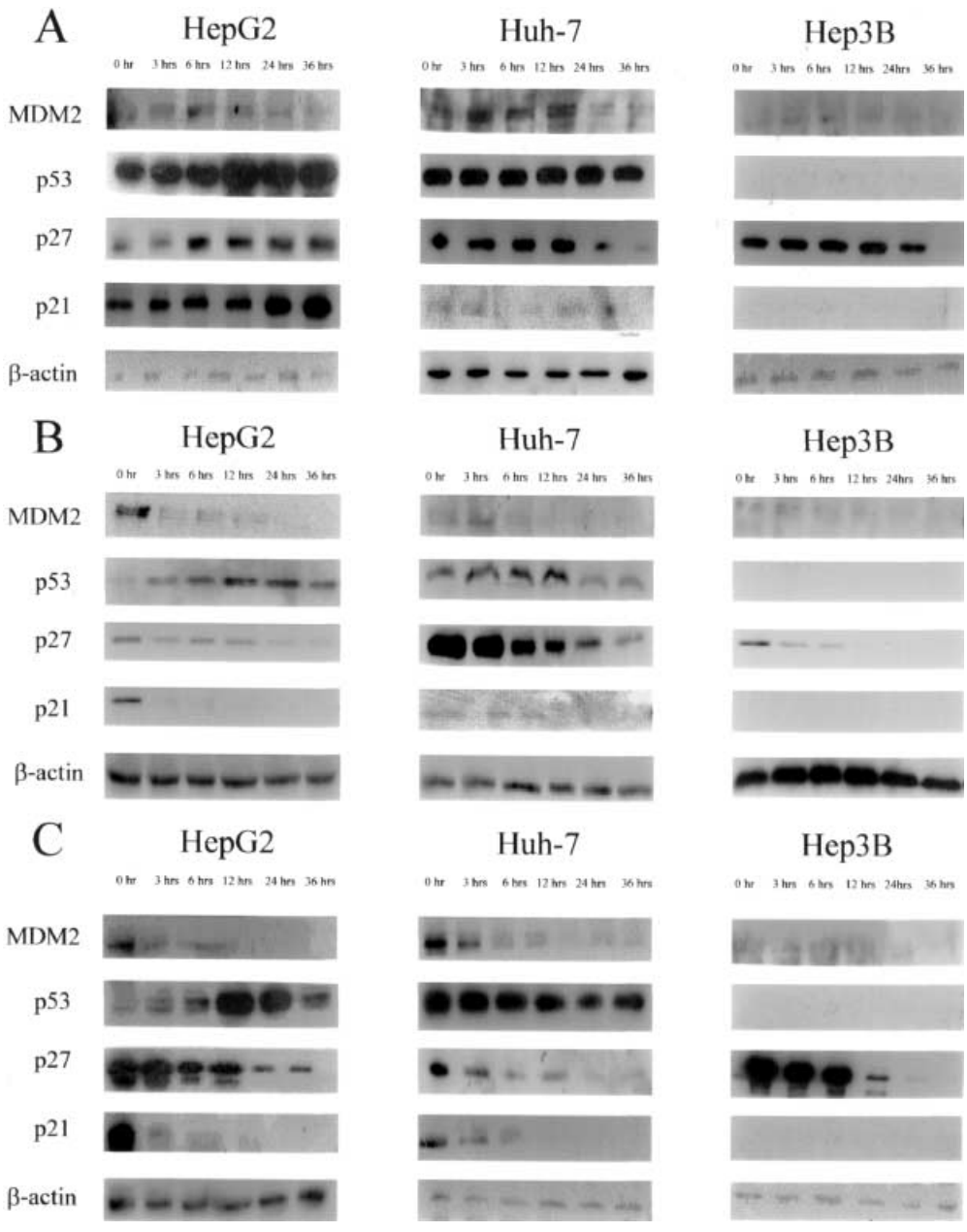

Fig. 5A–C Assessment of MDM2, p53, p27, p21 and *β*-actin (for protein level reference) in HepG2, Huh-7 and Hep3B cells after DOX treatment by Western blotting. The proteins were extracted before and 3, 6, 12, 24 and 36 h after DOX treatment at (**A**) IC_{10}, (**B**) IC_{50} and (**C**) IC_{90}

to the p53-MDM2 autoregulatory feedback loop [45]. It has been shown that induction of p53 upon DNA damage leads to *p53*-dependent upregulation of MDM2 until activation of *p53*-dependent target genes such as *p21* and *GADD45* is completed [1]. In contrast to the above observations, in the present study, downregulation of MDM2 independently of *p53* status was observed. Our findings therefore suggest that downregulation of MDM2 might be independent of p53 status and the p53-MDM2 autoregulatory feedback loop.

p21 was downregulated in Huh-7 and Hep3B cells but not in Hep3B cells, in which no p21 protein was detected because of the deleted *p53*. In HepG2 cells which has wild-type *p53*, it is believed that upregulated p53 protein levels in response to DNA-damaging agents transactivate its downstream target genes such as *p21* and *GADD45* before activation of *MDM2* [20, 21]. In HepG2 cells, apart from an upregulation of p21 after DOX treatment at IC_{10}, downregulation of p21 was observed with all other dosages of DOX. So, in HepG2 cells, upregulation of p53 was associated with downregulation of MDM2, p27 and p21 proteins, and there was G_1 arrest at IC_{50}. This observation is consistent with a previous report that, upon stimulation by DNA-damaging agents, there is downregulation of p21 protein accompanied by G_1 arrest [44]. Like p21, p27 is a cyclin-dependent kinase inhibitor which is responsible for arresting cells in the G_1 and G_2 phases [42]. In Hep3B and Huh-7 cells, there was a dose- and time-dependent decrease in p27 protein levels after DOX treatment.

In HepG2 cells, p27 was upregulated by treatment with DOX at IC_{10}, was decreased after a transient rise at IC_{50}, and was downregulated at IC_{90}. The response was somewhat similar to that of p21. Therefore, the findings suggest that p21 and p27 could be regulated independently of *p53*.

To conclude, DOX may confer different chemosensitivity on different hepatoma cell lines with different *p53* status. At low doses of DOX, but not at high doses, p53-dependent activation of p21 and p53-independent activation of p27 occurred. The contrasting relationships between chemosensitivity and *p53* status and expression suggest that DOX-induced apoptosis and cell death involve pathways independent of *p53*.

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