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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 G1 arrest 24 h after drug administration and upregulation of p53 protein level in a time-dependent manner. In Hep3B cells (deleted p53), G2/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 G1 nor G2 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 peucetius [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 perinuclear 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
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 G1 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% CO2. 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 IC50 (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 IC10, IC50, and IC90

To determine the cytotoxic effects of DOX on the hepatoma cell lines, the MTT assay was performed. The MTT assay allowed the determination of IC10, IC50, and IC90 from the dose response curves using multiple doses of DOX. IC10, IC50, and IC90 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 cytomtery. The cells were plated at a low density (5x10^3/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 Na4P2O7 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 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...