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Evaluation of an *In vitro* Approach to the Prediction of *In vivo* Effects on Multidrug Resistance in Human Hepatoma Cells

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Abstract

Objective: Multidrug resistance poses a major challenge to effective cancer chemotherapy. The present study evaluated whether a cell-based *in vitro* assay could provide a relevant screening system for agents with the potential to overcome cancer cell drug resistance.

Methods: We used a drug known to overcome doxorubicin resistance (verapamil) as a model compound and investigated its effects *in vitro* using three-dimensional Cellbed cultures of human hepatoma HepG2 cells, and *in vivo* using a HepG2 xenograft mouse model. In addition, we examined the expression of multidrug resistance protein 1 *in vitro* and *in vivo* using immunostaining.

Results: The half-maximal inhibitory concentration (IC_{50}) of doxorubicin was higher for HepG2 Cellbed cultures than in those cultured as a two-dimensional monolayer; these IC_{50} values were reduced by verapamil. Verapamil inhibited doxorubicin efflux from HepG2 Cellbed cultures. Doxorubicin resistance was also reduced by verapamil *in vivo*.

Conclusion: The three-dimensional Cellbed culture system could provide a promising cell-based *in vitro* assay system for the screening of agents that can overcome hepatic cancer cell drug resistance *in vivo*.

Keywords: Multidrug resistance; 3D culture; Silicate fiber scaffold; Cellbed; Human hepatoma; MDR1; Doxorubicin; Verapamil

Introduction

One of the major obstacles in the chemotherapeutic treatment of solid tumors is intrinsic or acquired multidrug resistance (MDR) [1]. MDR is associated with increased drug efflux from cancer cells, and is mediated by certain proteins such as P-glycoprotein (also known as MDR1) and MDR-associated protein [2]. MDR1 is primarily expressed in epithelial cells such as those of the small intestine, liver, kidney, and blood–brain barrier [3].

There is evidence suggesting that the use of small-molecule compounds that competitively inhibit drug transport by MDR1 is an effective strategy for overcoming MDR in cancer [4]. Various compounds including cyclosporine A, diltiazem, FK-506, and verapamil (VRP)-a calcium channel blocker-inhibited active drug efflux and restored the sensitivity of MDR cells to anticancer agents [5-9].

Cancer cell-based assay systems that reflect the *in vivo* MDR characteristics of the cells can be useful to screen for candidate drugs that can overcome MDR. Three-dimensional (3D) cultures have been investigated for their potential to maintain the biological characteristics of cancer cells [10,11], because drug resistance due to efflux activity cannot be modelled effectively using more traditional two-dimensional (2D) monolayer cultures [12]. In a previous study, we demonstrated that HepG2 human hepatic cancer cells formed spheroids on a poly-L-glutamic acid-coated dish and that their doxorubicin (DOX) efflux activity was higher than that of cells cultured in monolayers due to a higher expression of MDR1 [12]. Moreover, the amount of MDR1 per cell in spheroids was comparable to that of hepatic tumour tissue. The half-maximal inhibitory concentration (IC_{50}) of DOX was higher in spheroids than in a HepG2 monolayer, whereas both types of culture showed a similar IC_{50} for 5-fluorouracil, which is not exported by MDR1. These results suggest that 3D spheroid hepatic cancer cell cultures provide useful cell-based *in vitro* assay systems to determine the IC_{50} values of anticancer agents. However, it can be challenging to prepare spheroids of uniform size under conditions of sufficient medium renewal, which is required for these cell-based *in vitro* assays [13]. To address this, we investigated whether the MDR of HepG2 cells could be reproduced using Cellbed, a silicate fiber scaffold [14]. Cellbed is commercially available and provides a multi-cell layer-type 3D culture system, in which it is easier to control the HepG2 cell layer size, than in spheroids. We succeeded in identifying greater DOX resistance using Cellbed culture of HepG2 cells than when using a 2D monolayer culture. Furthermore, VRP reduced the IC_{50} of DOX in the 3D Cellbed culture, which suggested an *in vitro* reduction in DOX resistance [15]. However, to explore the efficacy of this assay system for screening agents capable of overcoming MDR, it is important to evaluate their effects on drug resistance in human hepatoma cells *in vitro* and *in vivo*.

Therefore, the present study investigated the effects of VRP on the IC_{50} of DOX for HepG2 and efflux activity *in vitro* using 3D Cellbed culture, and on DOX resistance *in vivo* using xenograft mice models of hepatoma. Furthermore, immunostaining was used to measure MDR1 protein expression *in vitro* and *in vivo*.

Materials and Methods

Cells and culture medium

Human hepatoma HepG2 cells obtained from the RIKEN Bio Resource Center (Tokyo, Japan) were subcultured as a monolayer in minimum essential medium (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA), 100 U/mL penicillin (Meiji Seika Pharma, Tokyo, Japan), and 100 U/mL streptomycin (Meiji Seika Pharma, Tokyo, Japan) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

3D culture

HepG2 cells were cultured using Cellbed™ (Japan Vilene Company, Ltd.).

Chemicals

DOX and VRP were obtained from Wako Pure Chemical Industries (Osaka, Japan). DOX was dissolved in sterile water to a stock concentration of 10 mM. VRP was dissolved in sterile water at a stock concentration of 200 mM.

Assessment of DOX IC_{50}

The DOX IC_{50} values for HepG2 cells were determined using the WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay (Cell Counting Kit-8; Dojindo Laboratories, Kumamoto, Japan). For 3D cultures, HepG2 cells (5.0×10^4/well) were inoculated in Cellbed 96-well plates and cultured in a 5% CO_2 humidified incubator at 37°C for 7 days. For monolayer cultures, HepG2 cells (2.0×10^3/well) were inoculated in standard 96-well plates (Sumitomo Bakelite, Tokyo, Japan) for 7 days. Cells were cultured for another 48 h after adding DOX (0.1-300 μM) in the presence or absence of 100 μM VRP. WST-8 solution (180 μL medium and 20 μL WST-8 reagent) was added to the plates, followed by incubation for 30 min. Absorbance was measured at a wavelength of 450 nm on a VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA). The DOX IC_{50} was determined by plotting the logarithm of drug concentration *vs.* growth rate (percentage of control) of treated cells.

DOX efflux assay

After growing HepG2 cells for 7 days in Cellbed, the medium was removed and the cells were incubated with fresh medium containing 50 μM DOX for 1 h. After the DOX-containing medium was removed, the cells were washed with PBS twice to remove free DOX. The cells were then incubated for 3 h with medium containing 100 μM VRP or medium alone. Aliquots of the conditioned media were collected at 30-min intervals and analyzed using a fluorescence spectrophotometer (F-2000, HITACHI, Tokyo, Japan) at excitation and emission wavelengths of 448 nm and 562 nm for DOX. In addition, the number of cells in each well was determined using the WST-8 assay.

Assessment of drug resistance *in vivo*

The mice were handled in accordance with the guidelines for animal experimentation specified by Japanese law. The animal studies were approved by the Committee on Animal Research of Sojo University. Female NOD/SCID/Jak3null (NOJ) mice (5-weeks-old) were provided by Dr. Seiji Okada (Center for AIDS Research, Kumamoto University, Japan) [16]. NOJ mice exhibit a complex type of hyperimmune deficiency; they lack complement and show macrophage and dendritic cell dysfunction (NOD mouse phenotype), they are deficient in mature T, B, and natural killer cells (Scid mouse phenotype), and show a natural killer cell deficiency derived from the Jak3null mouse. HepG2 cells (5.0×10^6 cells) suspended in Matrigel (BD Co., Franklin Lakes, NJ, USA) were subcutaneously inoculated into the dorsal flank of NOJ mice. These animals were randomized into 4 groups: control (n=9, physiological saline i.p. and i.v.); VRP-treated group (n=7, 25 mg VRP/kg/every 7 days i.p., physiological saline i.v.); DOX-treated group (n=7, physiological saline i.p., 1 mg DOX/kg/every 7 days i.v.); VRP+DOX combination group (n=7, 25 mg VRP/kg/every 7 days i.p., 1 mg DOX/kg/every 7 days i.v.). The doses of DOX and VRP were based on relevant literature [17-19]. Tumor volumes were calculated from (a) the smallest diameter and (b) the longest diameter using the following equation: tumor volume (V mm^3)=$0.5 \times a^2 \times b$. At the end point (28 days after cell transplantation), the subcutaneous tumors were removed and weighed.

Hematoxylin and eosin staining and immunostaining

In vitro specimens of 3D Cellbed cultures were taken on day 9 and *in vivo* specimens (tumors) were taken from the xenograft mouse model of human hepatoma on day 28. Each specimen was fixed using 10% formalin solution. The specimens were embedded in paraffin and sectioned (3 μm) using a microtome (RM2265; LEICA, Heidelberg, Germany). Sections were stained with hematoxylin and eosin and observed using an optical microscope (Olympus BX-53, Tokyo, Japan). Sections were also dewaxed in xylene and rehydrated through an ethanol series prior to immersion in Tris-EDTA buffer and antigen retrieval by autoclaving at 120°C for 20 min. They were then blocked in PBS containing 5% goat serum for 1 h at room temperature and washed twice with PBS before incubation with a control IgG (rabbit IgG isotype control, GTX35035; Gene Tex, Irvine, CA, USA) or an anti-MDR1 antibody (rabbit anti-P-glycoprotein antibody, ab170903; Abcam, Cambridge, MA, USA) overnight at 4°C; these antibodies were diluted 100-fold with PBS containing 3% BSA. The sections were then washed 3 times with PBS and endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 solution for 10 min at room temperature. The sections were incubated with a horseradish peroxidase-labeled secondary antibody (goat anti-rabbit IgG antibody, ab6721; Abcam) for 2 h at room temperature; this antibody was diluted 250-fold with PBS containing 3% BSA. The sections were then washed 3 times with PBS. Finally, the signal was detected by incubation with 3,3'-diaminobenzidine tetrahydrochloride. After counterstaining with hematoxylin, ethanol dehydration and xylene substitution were performed, and specimens were prepared for observation by optical microscope using mounting agent (Neo-Mount, 109016, Merck KGaA, Darmstadt, Germany).

Statistical analysis

Results are presented as mean ± standard deviation (SD). Data were statistically analysed using Student's t-test. Values of $p < 0.05$ were

considered to represent a statistically significant difference (one-sided test).

Results and Discussion

DOX IC$_{50}$ in 2D and 3D HepG2 cell cultures

The IC$_{50}$ of DOX for the growth of HepG2 cells was 5.18 µM in 2D monolayer culture and 55.7 µM for 3D Cellbed culture, indicating a higher drug resistance in the latter (Table 1). Furthermore, the IC$_{50}$ of DOX for 3D-cultured cells was decreased to 1.00 µM by treatment with VRP (100 µM), which had no effect on cell growth under these experimental conditions; in 2D culture, the DOX IC$_{50}$ was reduced to 0.60 µM in the presence of VRP (Table 1). These results suggested that the MDR of HepG2 cells and its reversal by VRP was recapitulated *in vitro* using the 3D Cellbed silicate fiber scaffold [14].

Culture	DOX IC$_{50}$ (µM)
2D VRP (-)	5.18 ± 1.76
2D VRP (+)	0.60 ± 0.08
3D VRP (-)	55.7 ± 3.87
3D VRP (+)	1.00 ± 0.07

Table 1: Effect of VRP on the DOX IC$_{50}$ in HepG2 cell cultures HepG2 cells were grown in two-dimensional monolayers (2D) or three-dimensional Cellbed cultures (3D). The DOX IC$_{50}$ values represent the mean ± SD of the indicated study groups (n=3), which were cultured in the presence (+) or absence (-) of 100 µM VRP.

DOX efflux from 3D-cultured HepG2 cells

Drug resistance arises due to drug efflux by MDR1 activity [12]. We examined the effects of VRP on DOX efflux from HepG2 cells in 3D Cellbed culture. After pre-incubation of the cells with 50 µM DOX at 37°C for 1 h, efflux was measured over time in the presence or absence of VRP. After 30 min, DOX efflux was apparently inhibited by VRP, as shown in Figure 1. This result suggests that the reduction of the DOX IC$_{50}$ in Cellbed culture by VRP reflects VRP-mediated inhibition of MDR1 activity.
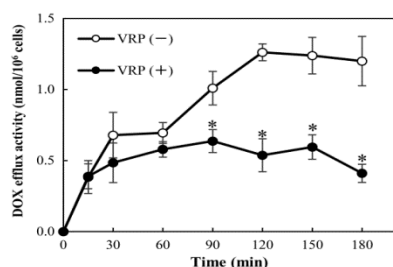

Figure 1: The time course of DOX efflux from HepG2 cells grown in 3D culture is shown in the presence (+) or absence (-) of VRP. Data represent the mean ± SD of the indicated group (n=5); *p<0.05 compared with the VRP (-) group.

Assessment of DOX anti-tumor activity in an *in vivo* subcutaneous xenograft mouse model of hepatoma

We initially examined the effects of treatment with DOX (1 mg/kg/every 7 days, i.v.) and/or VRP (25 mg/kg/every 7 days, i.p.) on body weight in a xenograft mouse model of hepatoma, generated by subcutaneous inoculation of HepG2 cells *in vivo*. Body weight decreased gradually in all study groups for 28 days after inoculation; no significant differences were observed between treatment and control groups at the doses tested, indicating that these doses of the compounds showed no severe side effects (Figure 2A).
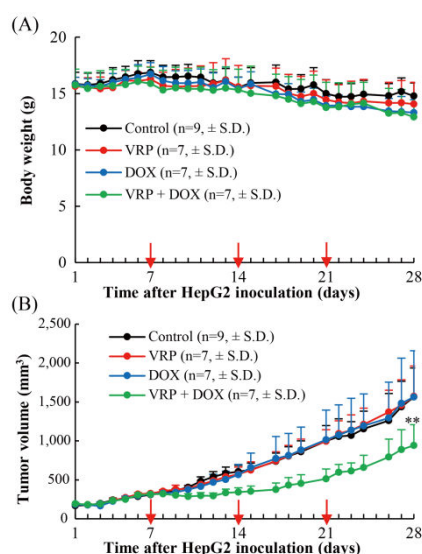

Figure 2: Body weights and tumor growth in the xenograft mouse model of hepatoma. Red arrows show the days when DOX (1 mg/kg) and/or VRP (25 mg/kg) were administered. (A) Mean body weights and (B) Tumor volumes of the indicated study groups. Data represent the mean ± SD of 7 or 9 animals; **p<0.01 compared with the other three study groups.

To examine the therapeutic effects of DOX and VRP, we measured tumor volumes in the xenograft mouse model of hepatoma. As shown in Figure 2B, the median tumor volume was 1564.9 ± 370.5 mm^3 in the control group, 1562.6 ± 594.3 mm^3 in the DOX-treated group, and 1571.5 ± 390.5 mm^3 in the VRP-treated group. There were no significant differences between the control, DOX, and VRP treatment groups. However, a marked reduction in tumor volume (942.4 ± 264.4 mm^3) was observed in the group treated with both DOX and VRP; this represented a 40% reduction compared to that in the control group (p<0.01 *vs.* control).

The subcutaneous tumors were resected from the anesthetized mice and weighed. Tumor images are shown in Figure 3A. These indicated that the combination of DOX and VRP produced therapeutic effects on this *in vivo* xenograft mouse model of hepatoma. Tumors of mice treated with both DOX and VRP were noticeably smaller than those of control, DOX-, or VRP-treated mice. The tumor weights are shown in Figure 3B. Significantly lower tumor weight was observed in mice treated with both DOX and VRP (1.07 ± 0.26 g) than in the control group (1.55 ± 0.36 g; p<0.05). These results indicate that DOX resistance was ameliorated by VRP in this *in vivo* model of hepatoma.
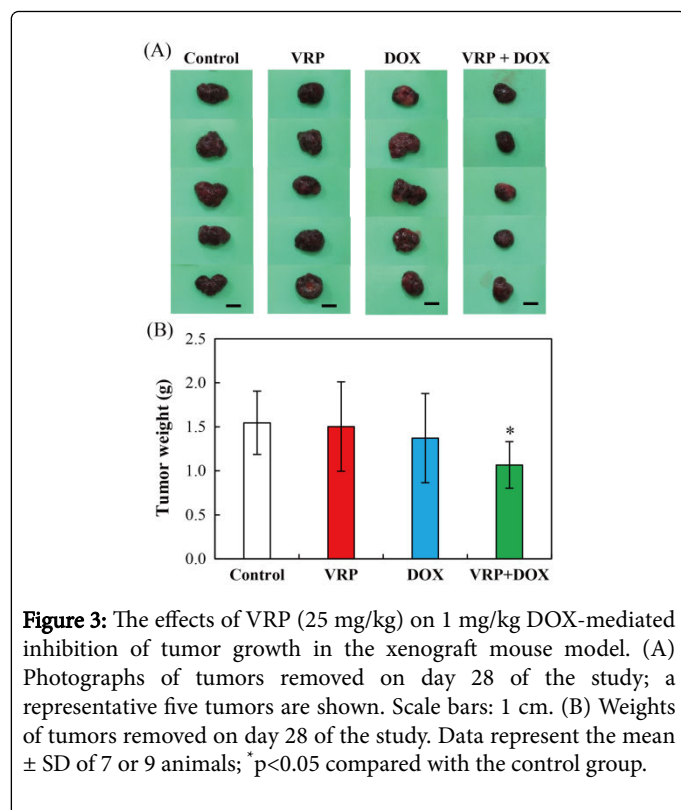
Figure 3: The effects of VRP (25 mg/kg) on 1 mg/kg DOX-mediated inhibition of tumor growth in the xenograft mouse model. (A) Photographs of tumors removed on day 28 of the study; a representative five tumors are shown. Scale bars: 1 cm. (B) Weights of tumors removed on day 28 of the study. Data represent the mean ± SD of 7 or 9 animals; *p<0.05 compared with the control group.

MDR1 expression by 3D-cultured HepG2 cells *in vitro* and by HepG2 xenografts *in vivo*

We evaluated MDR1 expression in 3D Cellbed cultures and in mouse xenografts using immunohistological analyses. The results are shown in Figure 4. Hematoxylin and eosin staining (Figures 4A and 4E) indicated that the cells in both specimens were viable and that the Cellbed cultures had formed three or four cell layers by day 9 of culturing (Figure 4A). IgG isotype staining indicated the non-specific background for MDR1 immunostaining in both specimens (Figures 4B and 4F). MDR1-positive cells were clearly observed in the 3D Cellbed cultures *in vitro* and in the xenografted tumors *in vivo* (Figures 4C and 4G). Furthermore, MDR1 proteins were partially localized at cell-cell adhesion points (Figures 4D and 4H). Our previous study indicated that MDR1 expression was higher in cells grown as 3D Cellbed cultures than in 2D monolayer cultures [12,15]; this may be related to the development of hypoxia in 3D tissue systems. The hypoxic environment in cancers such as hepatocellular carcinomas upregulates the expression of transcription factors such as hypoxia inducible factor-1α, which regulates MDR1 expression [20,21].

Tissue drug concentrations are determined by the pharmacokinetic properties of the drug after *in vivo* administration. The evaluation of drug efficacy, therefore, requires comprehensive animal experimentation [22]. The findings of the present study indicated that in relation to drug resistance, the same tendencies were observed *in vitro* using 3D Cellbed culture and *in vivo* using a xenograft mouse model of human hepatoma. These results suggest that 3D culture of human hepatoma using a silicate fiber scaffold could provide a promising cell-based *in vitro* assay system for the screening of agents capable of overcoming drug resistance *in vivo*, before conducting extensive preclinical animal experimentation.
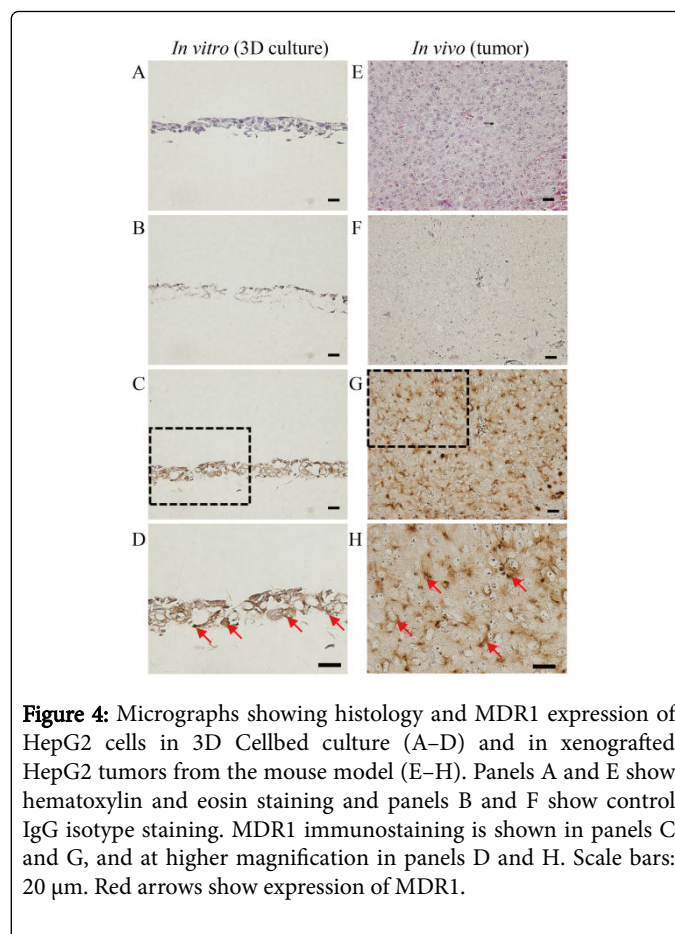

Figure 4: Micrographs showing histology and MDR1 expression of HepG2 cells in 3D Cellbed culture (A–D) and in xenografted HepG2 tumors from the mouse model (E–H). Panels A and E show hematoxylin and eosin staining and panels B and F show control IgG isotype staining. MDR1 immunostaining is shown in panels C and G, and at higher magnification in panels D and H. Scale bars: 20 μm. Red arrows show expression of MDR1.

Conclusion

In order to investigate whether a cell-based *in vitro* assay system could be used to screen for agents capable of overcoming drug resistance in cancer cells, we evaluated the effects of a compound known to reduce resistance (VRP) on the activity and efflux of DOX. These studies were conducted *in vitro* on 2D and 3D cultures of human hepatoma HepG2 cells and *in vivo* using a HepG2 xenograft mouse model of hepatoma. The DOX IC$_{50}$ was higher in 3D Cellbed cultures than in 2D monolayer cultures, and was reduced by VRP. The drug resistance of HepG2 cells appeared to depend on the MDR1 protein because VRP, which is known to inhibit MDR1 activity, inhibited DOX efflux from HepG2 cells. Furthermore, the administration of combined DOX and VRP *in vivo* overcame drug resistance in the xenograft mice model of human hepatoma. In addition, MDR1 expression was evaluated *in vitro* and *in vivo* using immunostaining. These findings indicate that 3D Cellbed cultures of human hepatoma HepG2 cells could provide a promising cell-based *in vitro* assay system for the screening of agents with the potential to overcome hepatic cancer cell drug resistance *in vivo*.

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