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Research Article

Induction of Drug Resistance in Human Hepatoma Cells Cultured on a Silicate Fiber-Based 3D Scaffold

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Abstract

Intrinsic or acquired multidrug resistance (MDR) of cancer cells is one of the major obstacles in the chemotherapeutic treatment of solid tumors. A cell-based *in vitro* system that reflects *in vivo* MDR characteristics of cancer cells can serve as a model for investigating the underlying mechanisms and developing a strategy for overcoming this problem. To this end, we generated a three-dimensional (3D) tissue-like model in Cellbed, a silicate fiber scaffold, using HepG2 human hepatic cancer cells. The half-maximal inhibitory concentration (IC_{50}) of doxorubicin (DOX) for cells cultured in Cellbed was higher than that for cells cultures as a 2D monolayer; this was reduced by the MDR-reversing drug verapamil. Drug resistance is presumed to depend on MDR1 protein, which exports DOX in hepatic cancer cells and may be related to the development of hypoxia in 3D-tissue systems as well as activation of hypoxia inducible factor-1. Hypoxia inducible factor-1, along with MDR1, was expressed at a higher level in cells grown in the 3Das compared to that in the 2D culture, as determined by immuno cytochemistry and western blotting. Thus, 3D Cellbed cultures of hepatic cancer cells reflect the drug resistance of cancer cells *in vivo* and are a promising cell-based *in vitro* assay system for examining the IC_{50} of anticancer agents. The system can also be used to screen for inhibitors of MDR1 activity that can overcome the MDR in cancer cells.

Keywords: Celled; Cell-based assay system; Doxorubicin; Hepatic cancer cells; 3D culture; Multidrug resistance; Verapamil

Introduction

One of the major obstacles in the chemotherapeutic treatment of solid tumors is intrinsic or acquired Multi Drug Resistance (MDR) in cancer cells [1]. MDR is associated with increased drug efflux from cancer cells, which 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]. Additionally, the hypoxic environment in cancers-including hepatocellular carcinoma-induces the up regulation of transcription factors such as hypoxia inducible factor (HIF)-1α that regulate MDR1 transcription [4,5].

There is evidence suggesting that targeting of MDR1 with small-molecule compounds that competitively inhibit drug transport by MDR1 is an effective strategy for overcoming MDR in cancer [6]. Various compounds including cyclosporine A, diltiazem, FK-506 and verapamil (VER)-a calcium channel blocker-inhibited active drug efflux and restored the sensitivity of MDR cells to anticancer agents [7-11].

Cancer cell-based assay systems that reflect the *in vivo* MDR characteristics of the cells can be useful for screening candidate drugs that can overcome MDR. Three-dimensional (3D) cultures have been investigated for their potential to maintain the biological characteristics of normal and cancer cells [12]. For example, rat hepatocytes has been shown to self-assemble into multicellular spheroids that maintain liver-specific activities such as albumin synthesis, urea genesis, and drug metabolism over a period of weeks [13,14], properties that are lost in a conventional monolayer culture. Multicellular spheroids of cancer cells also provide excellent 3D *in vitro* models that facilitate investigations into the response to anticancer drugs and the underlying mechanisms [15].

In a previous study, we demonstrated that HepG2 human hepatic cancer cells formed spheroids on a poly-L-glutamic acid-coated dish and that doxorubicin (DOX) efflux activity of cells thus cultured was higher than in monolayers due to higher expres-

sion of MDR1 [16]. Moreover, the amount of MDR1 per cell in spheroids was comparable to that in hepatic tumor tissue. Consequently, the half-maximal inhibitory concentrations (IC_{50}) of DOX was higher in spheroid as compared to monolayer HepG2 cells, whereas the IC_{50} of 5-fluorouracil, which is not exported by MDR1, was similar in both types of culture. These results suggest that 3D spheroid culture of hepatic cancer cells is a useful cell-based *in vitro* assay system for determining IC_{50} values of anticancer agents. However, one of the critical challenges is the difficulty in preparing spheroids of uniform size under conditions of sufficient medium renewal, which is required for the cell-based *in vitro* assay [17].

In the present study, we investigated whether the MDR of HepG2 cells can be reproduced by Cellbed, a silicate fiber scaffold [18]. Cellbed is a commercially available and multi-cell layer-type 3D culture system, in which it is easier to control cell layer size in HepG2 cells as compared to spheroids. We also compared the effects of the MDR-reversing drug verapamil on the IC_{50} of DOX for HepG2 cells in the 3D Cellbed and 2D monolayer cultures. It was previously reported that 13 human tumor cell lines including HepG2 showed up regulation of the nuclear factor (NF)-κB-regulated genes B cell lymphoma-2, cyclooxygenase-2, and vascular endothelial growth factor when grown in a 3D Cellbed culture than as a 2D monolayer [18]. NF-κB as well as HIF-1α expression is modulated by the hypoxic environment in tumor tissues [19]; we therefore compared the levels of HIF-1α as well as MDR1 in HepG2 cells grown in the two culture systems.

Materials and methods

Cells and Culture Medium

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

Chemicals

DOX and VER were obtained from Wako Pure Chemical Industries (Osaka, Japan). DOX was dissolved in sterile water at a final concentration of 10 mM. VER was dissolved in sterile water at a final concentration of 1 mM and diluted with Phosphate-Buffered Saline (PBS). Working solutions were prepared by dilution with sterile water.

3D Cellbed Culture

Cellbed was supplied by Japan Vilene Company (Tokyo, Japan). HepG2 cells were seeded at 3.0×10^5/well in 0.5 ml of MEM with 10% FBS in Cellbed 24-well plates and grown for 48 h at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The medium was changed at intervals of 48h and the cells were cultured for 7 days to allow formation of 3D spheroids.

Scanning Electron Microscopy (SEM)

Formaldehyde and glutaraldehyde-fixed samples were rinsed three times in PBS and then dehydrated in a graded series of ethanol (10%, 50%, 60%, 70%, 80%, 90%, and 100%).The samples were then transferred to t-butyl alcohol, lyophilized, and sputter-coated with platinum and palladium before visualization with a Miniscope TM3030 (Hitachi, Tokyo, Japan).

Assessment of DOX IC_{50}

The IC_{50} of drugs used to treat HepG2 cells was determined with 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, 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, 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). WST-8 solution (medium:WST-8 reagent= 180:20 μl) 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 IC_{50} of DOX in the presence or absence of 100μM VER [20] was determined by plotting the logarithm of drug concentration vs. growth rate (percentage of control) of treated cells. Data were analyzed with the Student's test. $P< 0.05$ was considered significant.

Confocal Laser scanning Microscopy

For 3D cultures, cells (3.0×10^5/well) were inoculated in Cellbed 24-well plates and cultured in a 5% CO_2 humidified incubator at 37°C for 7 days. For monolayer cultures, cells (2.0×10^4/dish) were inoculated in 35-mm glass-bottomed dishes (MatTek Corporation, Ashland, OR, USA) for 7 days. Cells were washed twice with PBS and fixed in 4% paraformaldehyde in PBS for 10 min. After washing with PBS, cells were incubated in PBS containing 1% Triton-X 100 for 30 min, washed twice in PBS with 0.01% Tween 20 (PBS-T), then blocked in PBS containing 10% goat serum and 0.1% Tween 20 for 30 min. After two washes in PBS-T, cells were incubated with mouse monoclonal anti-HIF-1α (1:200) (ab113642; Abcam, Cambridge, UK) or rabbit monoclonal anti-MDR1/ABCB1 (E1Y7B) (1:800) (#13342; Cell Signaling Technology Japan, Tokyo, Japan) antibodies in PBS-T for 2 h. After washing twice with PBS-T, cells were incubated with goat anti-mouse (1:250) (ab96879; Abcam) or anti-rabbit (1:250) (#4412;

Cell Signaling Technology Japan) IgG in PBS-T for 60 min followed by two rinses with PBS. After incubation in PBS containing 2 μM TO-PRO-3 (Life Technologies) for 10 min and washes in PBS, cells were visualized with a confocal laser scanning microscope (TCS-SP; Leica, Heidelberg, Germany).To visualize the cytoskeleton (actin fibers) and nuclei, fixed cells were incubated with 0.165 μM rhodamine phalloidin (Life Technologies) and 2 μM TO-PRO-3, respectively. Semi-quantitative analysis of the fluorescence intensity corresponding to MDR1 per cell was performed from the randomly selected confocal images using Image J software (National Institutes of Health, Bethesda, MD, USA).

Western Blot Analysis

For 3D cultures, cells (3.0×10^5/well) were inoculated in Cellbed 24-well plates and cultured in a 5% CO_2 humidified incubator at 37°C for 7 days. For monolayer culture, cells (2.0×10^5/dish) were inoculated in a standard 100-mm dish (Corning Inc., Corning, NY, USA) for 7 days. Cells were scraped from the dish and washed twice with PBS, then incubated for 5 min on ice in radioimmunoprecipitation assay buffer (Wako Pure Chemical Industries) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). After centrifugation at 1000 rpm for 5 min at 4°C, the protein concentration in the supernatant was measured with a Bradford protein assay kit (Takara Bio, Otsu, Japan). Aliquots of supernatant containing equal amounts of protein (50 μg) were incubated in NuPAGE lithium dodecyl sulfate sample buffer and reducing agent (both from Life Technologies) for 30 min at 70°C, then separated by electrophoresis on a NuPAGE Novex 4%–12% bis-Tris gel (Life Technologies). Proteins were transferred to a polyvinylidene difluoride membrane (iBlot Transfer StackMini; Life Technologies), which was incubated with Western Blot Blocking Buffer (Takara Bio) in TBS-T for 1 h at 25°C and then washed three times with TBST for 10 min each. The membrane was then probed with mouse monoclonal anti-HIF-1α (1:200) and mouse monoclonal anti-β-actin (1:1000) (SAB1305554-40TST; Sigma-Aldrich) antibodies for 24 h at 4°C, followed by incubation with horseradish peroxidase-conjugated goat anti-mouse IgG (1:5000) (sc-2005; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 24 h at 4°C. Immunoreactivity was visualized with Enhanced Western Blot Quant HRP Substrate (Takara Bio) according to the manufacturer's protocol and Lumi Vision PRO (Aisin Seiki, Aichi, Japan). Protein bands were semi-quantified using Image J software and intensity values were normalized to that of β-actin.

Results and Discussion

Generation of 3D HepG2 Cell Structures in the Cellbed Silicate Fiber Scaffold

HepG2 cells formed 3D structures when cultured in the Cellbed silicate fiber scaffold, as determined by SEM (Figure 1A). Cells were attached within and on the surface of the silicate fiber as well as to other cells, and had a cuboidal morphology (Figure 1B). Images of the cells were obtained in the X-Y plane and Z direction, using a confocal laser scanning microscope (Figure 1C, D), and revealed that the cells proliferated into Cellbed in two or three cell layers and engaged in cell-cell interactions. Although multi cellular spheroids of cancer cells also provide excellent 3D *in vitro* models [15], it is difficult to prepare spheroids of uniform size, especially for proliferating cancer cells [17]. On the other hand, it appears to be easier to control cell layer size of HepG2 cells cultured in Cellbed silicate fiber scaffold as compared to spheroids, which is required for the cell-based *in vitro* assay.

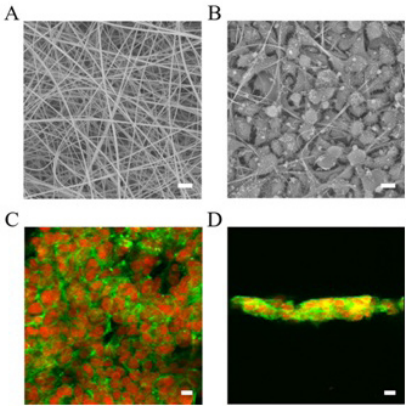

Figure 1: Visualization of HepG2 cells grown on Cellbed. (A) SEM observation of the Cellbed silicate fiber scaffold. (B) Formation of 3D structures by HepG2 cells grown on Cellbed (day 9 of culture). Confocal laser scanning micrographs of HepG2 cells grown on Cellbed (day 7 of culture). Shown are images of cells in the X-Y plane (C) and Z direction (D). Nuclei were stained with TO-PRO-3 (red) and the actin cytoskeleton was stained with rhodamine phalloidin (green). Scale bars: 10 μm.

Determination of DOX IC_{50}

Determination of IC_{50} is generally performed in cells grown as a monolayer in a 96-well plate. Drug efflux activity of cancer cells affects drug resistance and IC_{50}. In our previous work [16], we showed that the efflux activity and consequently, the IC_{50} of DOX export by MDR1 was lower in HepG2 cells grown as a 2D monolayer than as a 3D spheroid culture, resulting in a higher intracellular drug concentration under the former conditions. We therefore compared the IC_{50} of DOX for HepG2 cells grown in the two types of cultures. The IC_{50} of DOX was 2.69 μM for cells in the 2D monolayer and 52.6 μM for those in 3D Cellbed cultures, indicating a higher drug resistance in the latter (Figure 2). Furthermore, the high IC_{50} of DOX for cells grown as a 3D culture was decreased to 0.6 μM by treatment with the MDR-reversing drug VER (100 μM), which had no effect on cell growth under the experimental conditions. These results suggest that the MDR of HepG2 cells is recapitulated by culturing in the Cellbed silicate fiber scaffold [18].

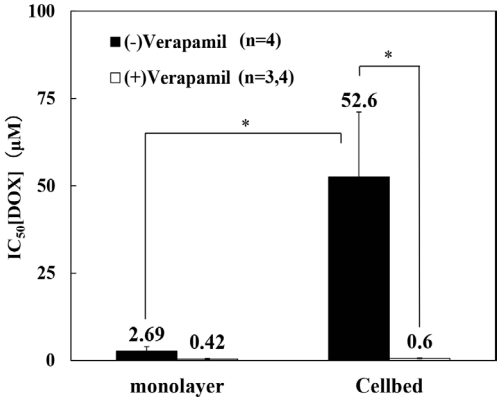

Figure 2: IC_{50} of DOX for HepG2 cells grown in 2D monolayer and 3D Cellbed cultures using VER. Error bars indicate standard error (n = 3 or 4) **P*< 0.05 (Student's test).

HIF-1α Expression in 2D and 3D Cultures

Given that the hypoxic tumor environment induces HIF-1α and consequently, MDR1 [4] expression, we compared HIF-1α levels in HepG2 cells grown as a 2D monolayer and in a 3D Cellbed culture. HIF-1α was more highly expressed in the latter, as determined by immunocytochemistry (Figure 3). A western blot analysis revealed a100-kD a band corresponding to HIF-1α (Figure 4A). The band intensity was higher for lysates of cells grown in the Cellbed as compared to the monolayer culture, and a quantitative analysis revealed a 1.94-fold difference in protein level (Figure 4B). Oxygen tension-as measured by a fluorescent oxygen probe -on the surface of sandwich-cultured hepatocytes (one cell layer) in a polystyrene dish was insufficient owing to the high oxygen consumption rate of hepatocytes [21]. In this study, the hypoxic tumor environment was reproduced in the 3D structure of cells grown in Cellbed, in which they formed two or three layers (Figure 1). Furthermore, the relationship between the hypoxic tumor environment and activation of transcription factors such as NF-κB and HIF-1α that modulate oncogenesis and anticancer drug sensitivity investigated in recent studies [19]. It is presumed that oxygen is in short supply for cells grown in the Cellbed owing to their 3D structure within the silicate fiber scaffold.

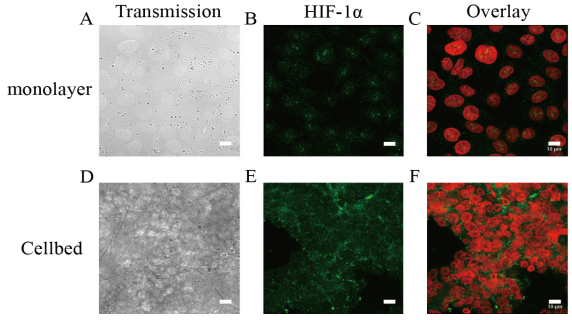

Figure 3: Confocal laser scanning micrographs of HIF-1α expression in HepG2 cells. Shown are (A, D) transmitted light view of monolayer (A) and Cellbed (D) cultures; (B, E) HIF-1α fluorescence alone (green); and (C, F) overlay of HIF-1α (green) and nuclei stained with TO-PRO-3 (red). Scale bars: 10 μm.

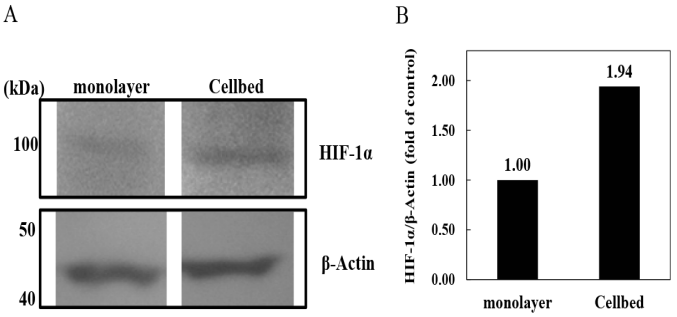

Figure 4: HIF-1α expression in HepG2 cells. (A) Western blot analysis of HIF-1α levels in HepG2 cells grown as a 2D monolayer and in a 3D Cellbed culture; β-actin was used as loading control. (B) Quantitative analysis of HIF-1α signal intensity relative to β-actin.

MDR-1 Expression in 2D and 3D Cultures

We also examined the expression of MDR1 -which is associated with MDR and is up regulated by HIF-1α [4]-by immuno cytochemistry. MDR1 expression was higher in cells grown as a 3D Cellbed culture as compared as those cultured as a 2D monolayer (Figure 5A-F); the protein was partially localized at cell-cell junctions (Figure 5G). The fluorescence intensity of MDR1expression was higher for the cells in the Cellbed as compared to the monolayer culture, and a semi-quantitative analysis of the fluorescence intensity per cell revealed a 4.2-fold difference (Figure 5H).

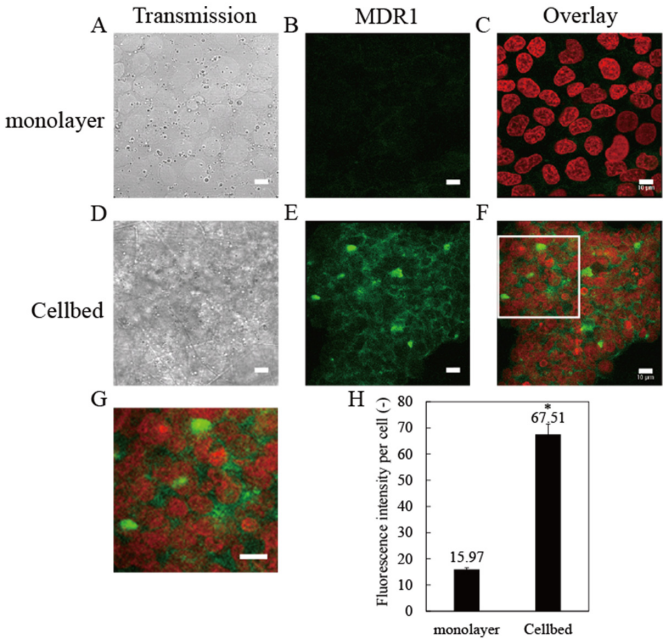

Figure 5: Confocal laser scanning micrographs of MDR1 expression in HepG2 cells. Shown are (A, D) transmitted light view of monolayer (A)

and Cellbed (D) cultures; (B, E) MDR-1 fluorescence alone (green); and (C, F) overlay of MDR1 (green) and nuclei stained with TO-PRO-3 (red). (G) Zoom in of F. Scale bars: 10 μm. (H) Semi-quantitative analysis of fluorescence intensity per cell corresponding to MDR-1 expression based on the images of B and E. Error bars indicate standard error (n=10) *P< 0.05 (Student's t test).

In conclusion, our results indicate that HepG2 cells cultured in a Cellbed silicate fiber scaffold can serve as a useful *in vitro* assay system for determining IC_{50} values of anticancer agents that reflects drug resistance in cancer cells. The system can also be used to screen MDR-reversing drugs that can provide more effective chemotherapy and improve prognosis in cancer patients.

Acknowledgments

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