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Resistance of multicellular aggregates to pharmorubicin observed in human hepatocarcinoma cells

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Abstract

The objective of the present study was to investigate the multicellular resistance of human hepatocarcinoma cells BEL-7402 to pharmorubicin. Cells (1 x 10^4) and 200 microcarrier Cytodex-3 beads were seeded onto a 24-well plate and cultured in RPMI 1640 medium. After the formation of multicellular aggregates, morphology and cell viability were analyzed by scanning electron microscopy, transmission electron microscopy and flow cytometry, respectively. The IC50 was determined by flow cytometry and MTT assay after the cells cultured in aggregates and monolayers were treated with pharmorubicin. The culture products exhibited structural characteristics somewhat similar to those of trabecular hepatocarcinoma *in vivo*. Among the microcarriers, cells were organized into several layers. Intercellular spaces were 0.5-2.0 µm wide and filled with many microvilli. The percent of viable cells was 87%. The cells cultured as multicellular aggregates were resistant to pharmorubicin with IC50 4.5-fold and 7.7-fold that of monolayer culture as determined by flow cytometry and MTT assay, respectively. This three-dimensional culture model may be used to investigate the mechanisms of multicellular drug resistance of hepatocarcinoma and to screen new anticancer drugs.

Key words
- Hepatocellular carcinoma
- Spheroids
- Drug resistance
- Microcarrier
- Epirubicin

Introduction

Intrinsic or acquired resistance to chemotherapeutic drugs is one of the major obstacles in the treatment of solid tumors. A number of possible mechanisms have been proposed to account for drug resistance (1). Previous research demonstrated that tumor cells cultured in three-dimensional (3-D) aggregates were resistant to cytotoxic drugs. The resistance was lost when the cells were disaggregated and cultured in monolayers. However, resistance could be acquired again when cells were cultured in 3-D aggregates (2,3). Many kinds of tumor cells become resistant to cytotoxic drugs when cultured in 3-D aggregates. Three-dimensional culture makes it possible to directly relate structure to function and to reveal the response of cells to chemotherapeutic drugs similar to that observed *in vivo*. Three-dimensional culture of hepatocarcinoma cells has been reported (4-7), but hepatocarcinoma cells with different characteristics need to be investigated.

Previously, 3-D culture of hepatocarcinoma was used to study the cytotoxic effects of alcohol and to determine the function of hepatoma cells. In the present study, human hepatocarcinoma cells (BEL-7402) were cultured with microcarrier beads (Cytodex-3) to establish a 3-D model and to investigate multicellular drug resistance to pharmorubicin.

Material and Methods

Cell line and cell culture

BEL-7402 cells were derived from a specimen obtained from a 53-year-old male patient with hepatocarcinoma in 1974. The cell line was established at the Shanghai Institute of Cell Biology, Academia Sinica. The cells were cultured in RPMI 1640 medium (Gibco BRL, Rockville, MD, USA) supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA), 100 U/ml penicillin and 100 U/ml streptomycin in the presence of 5% CO_2 and 95% air, at 37ºC (8).

Three-dimensional culture

Microcarrier beads Cytodex-3 (Sigma, St. Louis, MO, USA) were allowed to swell in Ca^{2+}- and Mg^{2+}-free PBS and then sterilized at 110ºC for 30 min. The beads were washed twice with sterile PBS, pH 7.4, before culture. BEL-7402 cells were harvested freshly by treatment with 0.25% trypsin containing 2% EDTA. Cells (1 x 10^4) and 200 microcarrier beads per well were seeded onto a 24-well plate and cultured in the same medium. The plate was coated with 10% poly(2-hydroxyethyl methacrylate) (Sigma) to prevent cell adhesion.

Scanning electron microscopy

After a mean 10-day period of culture, the microcarrier beads were aggregated and covered with multilayer cells as observed with the phase contrast microscope. A further 4-5 days later, samples were washed with PBS, pH 7.4, and fixed with 2.5% glutaraldehyde (SPI-Chem, West Chester, PA, USA) in PBS, pH 7.4, for 1 h at room temperature. After three washes in PBS, they were post-fixed with 1.0% osmium tetroxide (Matthey, Materials Technology, Hertfordshire, England) in PBS for 1 h at room temperature followed by dehydration with a growing ethyl alcohol series (30, 50, 70, 80, 90 and 100%). The samples were then treated with iso-amylacetate for more than 2 h, dried to the critical point and coated with gold. In order to observe cross-sections of the aggregates, part of the aggregates were cleaved before being coated with gold. Finally, the samples were observed with a scanning electron microscope (JEOL, TSM-5600LB, Tokyo, Japan).

Transmission electron microscopy

Additional samples were fixed and dehydrated as described for scanning electron microscopy and embedded in Epon812 epoxy resin (Epon812 kit, SPI-Chem). Thin sections were prepared and examined with a transmission electron microscope (JEM-1230, Tokyo, Japan).

Flow cytometry

In order to determine whether apoptosis and necrosis occurred after 3-D aggregates formed for 4-5 days, cells freshly harvested by enzymatic dissociation were stained with propidium iodine (Sigma) and Annexin-V-FLUOS (Boehringer, Mannheim, Germany), and analyzed by flow cytometry (FACS-Calibur, Becton Dickinson, San Jose, CA, USA) according to the instructions of the manufacturers of the Annexin-V-FLUOS kit. Briefly, cells (1 x 10^6) were suspended in 100 µl labeling solution (20 µl Annexin-V-FLUOS-labeling reagent in 1000 µl HEPES buffer with 20 µl propidium iodide (50 µg/

ml) added). Samples stained only with propidium iodide or Annexin-V-FLUOS were used as single staining control. All samples were incubated at room temperature for 10-15 min in the dark. A control without staining was also prepared. Incubation buffer (400 µl) was added to the samples before flow cytometry. The data were analyzed with the CellQuest software (Becton Dickinson).

Cells cultured in monolayers and the 3-D aggregates mentioned above were treated with 1.25, 2.5, 5, 10 or 20 µM pharmorubicin (Pharmacia & Upjohn S.P.A., Peapack, NJ, USA) in the above culture medium for 30 h. Then the apoptosis and necrosis of 5 x 10^5 cells per sample were analyzed by the same method as above. The percentage of viable cells was determined and the IC50 was calculated by the regression method.

MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma) was dissolved in PBS at 5 mg/ml and sterilized by filtration. After treatment with 1, 2, 4, 8, 16 or 32 µM pharmorubicin for 30 h, the cells were freshly disaggregated by enzymatic dissociation and the cell number was determined with a hemocytometer. A cell suspension (100 µl) of each sample was added to a 96-well plate. The cell number per well was within the range of the standard curve. Stock MTT solution (10 µl per 100 µl medium) was added to all wells. After incubation in the presence of 5% CO_2 and 95% air at 37ºC for 6 h, the supernatant was discarded. Dissolved solution (100 µl) (17% Triton X-100, 6.45 mol/l dimethylformamide, 0.2 mol/l citric acid) was added to each well and mixed thoroughly to dissolve the dark blue crystals. After half an hour at room temperature to ensure that all crystals were dissolved, the plates were read with a MicroElisa reader at a wavelength of 570 nm. All samples were read in triplicate. Finally the percentage of viable cells was determined and the IC50 was calculated by the regression method.

Statistical analysis

Data are reported as means ± SEM. The chi-square test was used for statistical analysis ($P<0.01$).

Results

Morphology (scanning and transmission electron microscopy)

The aggregates were irregular with a diameter of up to 1.0-2.0 mm and composed of microcarriers and cells. The cells were oval spheroid or polyhedric with many microvilli on the surfaces and packed together densely. Cross-sections showed that cells were organized densely in multilayers and distributed among the microcarriers (Figure 1). Transmission electron microscopy showed that cells were polygonal and arranged in several layers. Adjacent cells were simply apposed and tight junctions were observed. The intercellular space was 0.5-2.0 µm wide and contained many microvilli which enabled the exchange of liquid medium through the aggregates. Cytoplasm was full of mitochondria (Figure 2).

Cell viability

Apoptosis and necrosis of the 3-D aggregates described above were analyzed with a flow cytometer. Cell viability was 87.77 ± 1.76%. Apoptosis and necrosis were 2.77 ± 0.52 and 11.11 ± 3.37%, respectively.

Sensitivity test

The sensitivity of the cells cultured in monolayers and 3-D aggregates to pharmorubicin was investigated by flow cytometry and MTT assay. Cell mortality in 3-D cul-

tures treated with various concentrations of pharmorubicin was higher than that of monolayer culture ($P<0.01$) (Figures 3 and 4). The IC50 for 3-D aggregates was 4.5-fold that of monolayer culture by flow cytometry and 7.7-fold by the MTT assay, indicating that BEL-7402 cells cultured in 3-D became resistant to pharmorubicin (Table 1).

Discussion

Three-dimensional cell culture had been

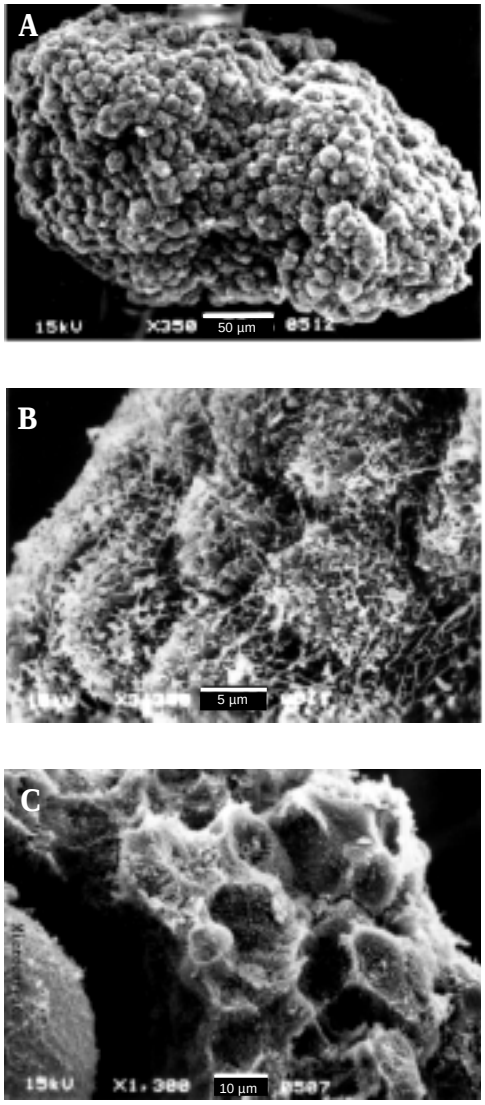

Figure 1. Scanning electron microscopy of 3-D culture of BEL-7402 cells. The 3-D structure lasted 4-5 days. *A*, The aggregates were irregular with a diameter up to 1.0-2.0 mm and covered with densely apposed cells (350X). *B*, Local amplification of aggregates (3,300X). The cells were spherical with many densely arranged microvilli. *C*, Cross-section of the aggregates (1,300X). The cells were polyhedric, arranged into multilayers and covered the microcarrier beads.

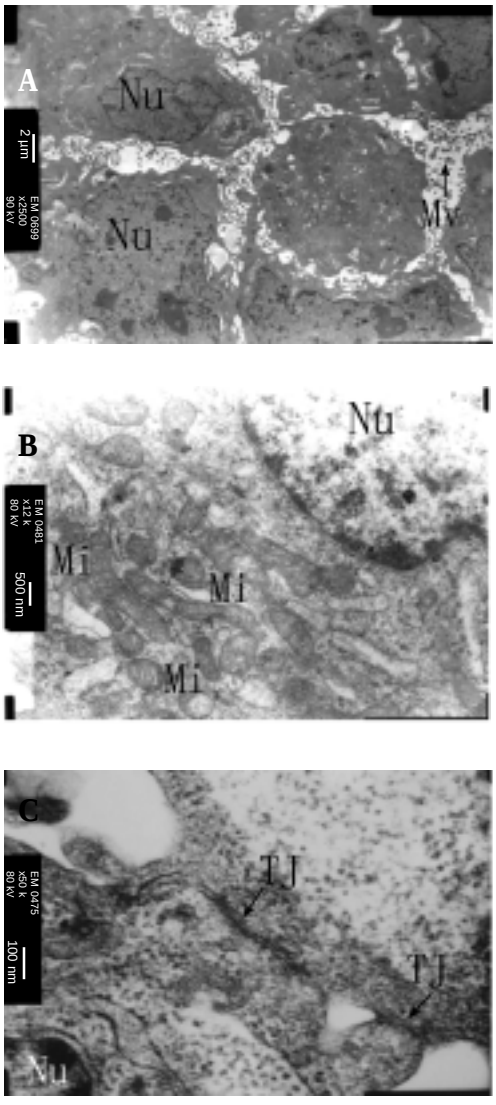

Figure 2. Transmission electron microscopy of 3-D culture of BEL-7402 cells. The 3-D structure formed for 4-5 days. *A*, Cells arranged in multilayers (2,500X). The intercellular space was 0.5-2.0 µm wide and filled with many microvilli (Mv). The nucleus (Nu) was large and irregular. *B*, Cytoplasm was rich in mitochondria (Mi) (12,000X). *C*, Adjacent cells were connected by tight junctions (TJ) (50,000X).

widely used in biomedical research (2,9-11). Many kinds of solid tumors *in vivo* and tumor cells cultured in 3-D *in vitro* exhibit intrinsic or acquired resistance to cytotoxic drugs, which is one of the major obstacles to clinical treatment. Various factors are involved in multicellular drug resistance, such as alterations in drug transport (12), drug metabolism (13), drug target (14), cellular repair mechanisms (15) and a decreased susceptibility to apoptosis (16). Understanding the mechanisms of multicellular drug resistance will contribute to the exploration of more efficient chemotherapy strategies. In this study, human hepatocarcinoma cells BEL-7402 were cultured with Cytodex-3 microcarrier beads to form 3-D aggregates. The results indicated that the cells were oval spheroid or polyhedric. Cells were arranged together densely in multilayers which were distributed among the microcarriers. *In vivo*, trabecular hepatocarcinoma is characterized by multilayers of hepatocarcinoma cells that are separated by hepatocytes or sinuses (17). In this model the multilayers of hepatocarcinoma cells were separated by microcarrier beads but not by sinuses. Multilayers of cells were distributed among the microcarrier beads and covered the aggregates, thus resembling, to a certain extent, the morphological characteristics of trabecular hepatocarcinoma *in vivo*. Tight junctions were found, and our previous study indicated that desmosomes were formed as well, properties that are essential for the integrity of the 3-D aggregates. The percent of viable cells was 87%. The presence of many mitochondria and microvilli suggested that the cells were in good condition. The cells turned out to be less sensitive to pharmorubicin than the cells cultured in monolayers. Our previous study indicated that after the formation of 3-D aggregates the percent of cells in the G0-G1 phase increased, while the percent of cells in the G2-M phase decreased. This suggests that more cells shift into a quiescent state (data not shown). However, the majority of conventional cytotoxic anticancer drugs preferentially kill cycling cells. The increase of the percent of quiescent cells and the decrease of efficiency of drug penetration into the center of the aggregates might result in a decreased sensitivity.

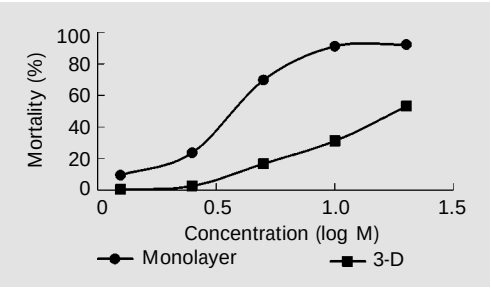

Figure 3. Sensitivity of BEL-7402 cells to pharmorubicin determined by flow cytometry. Cells cultured in monolayer and 3-D aggregates were treated with 1.25, 2.5, 5, 10 and 20 µM pharmorubicin for 30 h. Apoptosis and necrosis were then analyzed by cytometry and cell mortality was determined. The cells in monolayer culture were more sensitive to pharmorubicin ($P<0.01$, chi-square test).

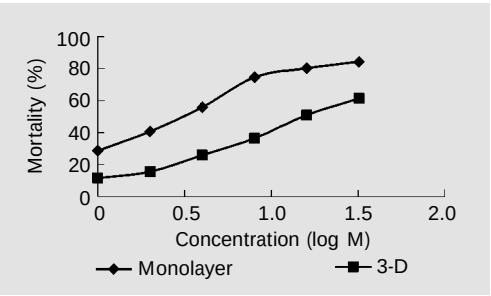

Figure 4. Sensitivity of BEL-7402 cells to pharmorubicin determined by the MTT assay. After treatment with 1, 2, 4, 8, 16 and 32 µM pharmorubicin for 30 h, the cells in 3-D aggregates and monolayer culture were cultured with MTT solution and cell mortality was determined. The cells in monolayer culture were more sensitive to pharmorubicin ($P<0.01$, chi-square test).

Table 1. IC50 (µM) of pharmorubicin applied to BEL-7402 cells analyzed by flow cytometry and MTT assay.

	Monolayer culture	3-D culture
IC50 flow cytometry	4.50 ± 0.68	20.16 ± 5.7
IC50 MTT assay	3.41 ± 0.63	26.30 ± 13.7

Data are reported as means ± SEM.

Understanding the mechanisms of multicellular drug resistance should improve clinical chemotherapeutic strategies. In terms of screening new drugs, if drugs demonstrate anticancer activity comparable to or greater than that seen in a monolayer culture, this could indicate more promising efficacy for

the treatment of solid tumors *in vivo*. Cells in this 3-D model were organized into a multilayer structure resembling, to a certain extent, the morphological characteristics of trabecular hepatocarcinoma *in vivo*. The cells cultured in 3-D aggregates were resistant to pharmorubicin compared with monolayer culture, a result that may contribute to research on multicellular drug resistance and to the screening of new anticancer drugs.

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