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Three-Dimensional, but not Two-Dimensional, Culture Results in Tumor Growth Enhancement after Exposure to Anticancer Drugs

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Previously, we have adapted a recently developed three-dimensional chemosensitivity test, the collagen gel droplet embedded culture drug sensitivity test (CD-DST), for evaluation of chemosensitivity of 12 anticancer drugs against colorectal adenocarcinoma, and surprisingly, it was found that tumor growth enhancement was occasionally observed even after exposure to anticancer drugs. In this study, the CD-DST was applied for human cervical carcinoma cell line HeLa-Ohio (HeLa) cells and its MDR1/P-glycoprotein-overexpressing subline, Hvr100-6 cells, and 12 anticancer drugs were assessed in terms of chemosensitivity and deterioration of tumor, and the results were compared with those by two-dimensional WST-1 assay. Growth enhancement was observed in Hvr100-6 cells, not in HeLa cells, for mitomycin C with the ratio of total volume of colonies in the treated group to that in the untreated group (T/C%) of 135.0%, doxorubicin with T/C% of 162.5% and cyclophosphamide with T/C% of 122.0%, and this was not observed in WST-1 assay. Multidrug resistance was detected both for CD-DST and WST-1 assay. The values of T/C% in CD-DST were comparable with or higher than those of the survival fraction (%) in WST-1 assay, and modification of WST-1 assay procedure gave similar results, suggesting a higher resistance in three-dimensional than in two-dimensional culture. Further investigations should be addressed to the association of MDR1/P-glycoprotein with tumor growth enhancement.

Chemosensitivity tests are understood to be useful especially in terms of optimization of cancer chemotherapy via the selection of anticancer drugs for an individual patient, and various tests have been developed for the last 20 years, including the subrenal capsule (SRC) assay (1), human tumor clonogenic assay (HTCA) (17,20,24), thymydine incorporation assay (TIA) (7,22), succinic dehydrogenase inhibition (SDI) assay (11), MTT assay (2,13), and histoculture drug response assay (HDRA) (4,12,23). However, these methods have not

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been adopted with a wide-ranging clinical base due to various problems listed below. Large specimens are required for HTCA and TIA. HTCA, SDI assay and MTT assay have often failed due to a low success rate of primary culture and/or contamination of fibroblasts. MTT assay and HDRA sometimes require a higher concentration of anticancer drugs than clinically achievable concentrations, suggesting that the mode of action might differ from the clinical situation (12,23). To overcome these issues, the collagen gel droplet embedded culture drug sensitivity test (CD-DST) has been developed in 1997 (8,9). By embedding and culturing tumor cells in collagen gel droplets, three-dimensional in vivo-like assessment is enabled with a relatively small number of cells, but with a higher success rate. Fibroblast contamination is experimentally ignored by the application of imaging analysis to select the targeted tumor colonies. Immediately thereafter, the predictability has been assured for various types of solid tumors, including breast cancer, lung cancer, gastric cancer, and carcinomas of the pancreas and biliary tract (10,14,25), and now the CD-DST is expected to be one of the most promising tests.

Previously, we have adapted the CD-DST for evaluation of chemosensitivity of 12 anticancer drugs in 25 Japanese patients with colorectal adenocarcinoma (15,16), and it was concluded that 1) the chemosensitivity was successfully evaluated for 64% (16/25) of patients, 2) the anticancer drugs were effective against the samples showing a relatively high growth rate, 3) gemcitabine hydrochloride (GEM) was more promising than 5-fluorouracil (5-FU), irinotecan hydrochloride (CPT-11), mitomycin C (MMC) and cisplatin (CDDP), which are often prescribed for colorectal adenocarcinoma, 4) MMC was more effective with relatively high mRNA expression of multidrug resistance-associated protein 2 (MRP2), but no such correlation was observed for multidrug resistant transporter, MDR1 and MRP1, and 5) the chemosensitivity against 5-FU, CPT-11, MMC and CDDP was independent of the genotypes of *MDR1* C3435T, *MRP1* G2168A and *MRP2* C-24T (C3972T), possibly due to no association with the growth rate of colorectal adenocarcinoma, nor mRNA expression of MDR1, MRP1 and MRP2 in colorectal adenocarcinoma.

In addition to these observations, surprisingly, tumor growth enhancement was occasionally observed even after exposure to anticancer drugs (15,16), that is, more than 120% enhancement was found for 14 of 137 CD-DST assessments and 5 cases attained more than 150% (15). It was discriminatively noted that 11 of 14 cases were on 2 patients. The patients were not characterized by gender and age, but mRNA expression (/GAPDH mRNA) of MDR1 (0.95, 0.59) and MRP2 (0.02, 0.00) was relatively lower than the average (±SD); 3.18±4.63 and 0.07±0.09, respectively, strongly suggesting the association of MDR1 and MRP2 with deterioration. In this study, the CD-DST was applied for human cervical carcinoma cell line HeLa-Ohio (HeLa) cells and its MDR1-overexpressing subline, Hvr100-6 cells (6,21) and 12 anticancer drugs were assessed in terms of chemosensitivity and deterioration, and the results were compared with those by two-dimensional, conventional WST-1 (modified MTT) assay.

MATERIALS AND METHODS

Materials. 7-Ethyl-10-hydroxycamptothecin (SN-38), an active metabolite of CPT-11, was a gift from Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan), docetaxel (TXT) was from Aventis Pharma Ltd. (Vitry sur Seine, France), GEM was from Eli Lilly & Co. (Indianapolis, IN, USA), and vindesine sulfate (VDS) was from Shionogi & Co., Ltd. (Osaka, Japan). 5-FU, MMC, doxorubicin hydrochloride (DXR), paclitaxel (TXL), cyclophosphamide monohydrate (CPA) and etoposide (VP-16) were purchased from Wako Pure Chemical

Industries, Ltd. (Osaka, Japan). CDDP and methotrexate (MTX) were from Sigma-Aldrich Co. (St. Louis, MO, USA). Other chemicals used were of the highest purity available.

Cells and cell culture. HeLa cells were obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan). HeLa cells (396-401 passage) were maintained in culture medium consisting of Dulbecco's modified Eagle's medium (D-MEM with glucose (4.5 g L^{-1}), L-glutamine (4 mM) and sodium pyruvate (1 mM); Cat. No. 12800-017, Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Lot. No. AGM7413, HyClone, UT, USA) and 100 mg L^{-1} kanamycin sulfate (Invitrogen Corp.). A vinblastine-resistant subline of HeLa, Hvr100-6, was established by stepwise increases of concentration of vinblastine sulfate in the culture medium (5 x 10^6 cells per 60 mm dish). FACS and RT-PCR analysis confirmed the induction of MDR1 in Hvr100-6 cells without alterations of related transporters, including MRP1, MRP2, and breast cancer resistance protein (BCRP) (6,21). Hvr100-6 cells (82-98 passage) were derived and maintained with 100 nM vinblastine sulfate. Both of these cell lines (4 and 12 x 10^4 cells cm^{-2}, respectively) were seeded into culture flasks (Nalge Nunc International, NY, USA), grown in a humidified atmosphere of 5% CO_2-95% air at 37°C, and subcultured every 3 or 4 days with 0.05% trypsin-0.02% EDTA (Invitrogen Corp.).

Chemosensitivity of HeLa and Hvr100-6 cells assessed by three-dimensional CD-DST. The CD-DST was conducted as described previously (8,9). Briefly, the Type I collagen solution (Cellmatrix type CD™, Nitta Gelatin Inc., Osaka, Japan), 10 x Ham's F-12 medium and a reconstitution buffer (Nitta Gelatin Inc.) were mixed together at a ratio of 8:1:1. The cell suspension prepared was added to the collagen mixture at a final concentration of 1 x 10^5 cells mL^{-1}. This collagen-cell mixture was dropped into 6-well plates (Nalge Nunc International) at a volume of 30 μL per collagen droplet, and subjected to gelation in a CO_2 incubator at 37°C for 1 h. In each well, 3 mL per well of DF medium™ (DF(10), Nissui Pharmaceutical Inc., Tokyo, Japan) containing 10% FBS (HyClone) was overlaid on day 0. After overnight incubation (on day 1), a test anticancer drug was added, at final concentrations of 1.0 μg mL^{-1} (7.69 μM) for 5-FU, 0.03 μg mL^{-1} (0.0750 μM) for SN-38, 0.03 μg mL^{-1} (0.0897 μM) for MMC, 0.2 μg mL^{-1} (0.667 μM) for CDDP, 0.02 μg mL^{-1} (0.0345 μM) for DXR, 0.1 μg mL^{-1} (0.116 μM) for TXT, 0.1 μg mL^{-1} (0.117 μM) for TXL, 0.3 μg mL^{-1} (0.660 μM) for MTX, 0.2 μg mL^{-1} (0.717 μM) for CPA, 0.4 μg mL^{-1} (1.33 μM) for GEM, 0.01 μg mL^{-1} (0.0117 μM) for VDS and 1.0 μg mL^{-1} (1.70 μM) for VP-16. The concentrations used for 5-FU, MMC, CDDP, DXR, VDS and VP-16 were those often used for CD-DST (10,14,25). The concentrations of SN-38, TXT, TXL, MTX, CPA and GEM were determined as the quotient of the area under the concentration-time curve by 24 hr, the exposure time for anticancer drugs in CD-DST. After 24 hr (on day 2), each well was washed twice with 3 mL per well of warmed complete Hank's balanced salt solution (HBSS, Cat. No. H-6136, Sigma-Aldrich Co.), and 4 mL/well of PCM-2 medium™ (Nitta Gelatin Inc.) was overlaid, and the cells were cultured for 7 days. On day 9, 50 μg mL^{-1} of neutral red solution was added and incubation was continued for 2 h. After removal of the solution, cells were fixed with 10% formalin buffered at neutral pH. The plates were immersed in water in a tray for 10 min without agitation, and then air-dried and subjected to evaluation by imaging analysis. The growth rate of the tumor cells was evaluated as the ratio of total volume of tumor colonies after the culture for 7 days to that before the culture in the untreated group, and the data where the growth rate was more than 0.8 were successfully adopted. The chemosensitivity was evaluated as the T/C% ; , the ratio of total volume of tumor colonies in the treated group to that in the untreated group. One untreated

group was set per five treated groups. For the prediction of clinical response, a T/C% less than or equal to 50% was considered effective and that greater than 60% not effective.

Chemosensitivity of HeLa and Hvr100-6 cells assessed by two-dimensional WST-1 assay. Antiproliferative effects were assessed in HeLa and Hvr100-6 cells by the WST-1 (tetrazolium salts) colorimetric assay using a Cell Counting Kit (Dojindo Laboratories, Kumamoto, Japan). Cells (1,000 cells per well) were seeded on 96-well plates (Nalge Nunc International) in 100 μL of DF medium™ (Nissui pharmaceutical Co., Ltd.) supplemented with 10% FBS (HyClone) on day 0, and the medium was exchanged to that containing a test anticancer drug at designated concentration described above on day 1. On day 4, the medium was exchanged to 110 μL of that containing WST-1 reagent solution (10 μL WST-1 solution and 100 μL the medium), and 3 h later, the absorbance was determined at 450 nm with a reference wavelength of 630 nm using a microplate reader (Sjeia Auto Reader II; Sanko Junyaku Co. Ltd., Tokyo, Japan). The chemosensitivity was evaluated as the survival fraction (%) ; , the ratio of cell number in the treated group to that in the untreated group.

In addition to the standard assay procedure related above, the chemosensitivity was evaluated under 1-day exposure condition, i.e., a test anticancer drug added on day 1 was removed by exchanging the medium with anticancer drug-free medium on day 2. The chemosensitivity was also evaluated under 1-day exposure condition without FBS, i.e., a test anticancer drug and FBS were removed by exchanging the medium with anticancer drug- and FBS-free medium on day 2.

RESULTS AND DISCUSSION

Table 1 lists the data on the chemosensitivity of 12 anticancer drugs assessed by two-dimensional WST-1 assay and three-dimensional CD-DST in HeLa and Hvr100-6 cells. In WST-1 assay, tumor growth enhancement was not observed. Relative resistance, defined as the ratio of the survival fraction (%) for Hvr100-6 cells to that for HeLa cells herein, was 43.7 for TXL, 22.8 for TXT, 10.2 for VDS and 2.4 for DXR, whereas it was almost unity for 5-FU, SN-38, MMC, CDDP, MTX, CPA and GEM. For CD-DST, growth enhancement was confirmed in Hvr100-6 cells, not in HeLa cells, for MMC with T/C% of 135.0%, DXR with T/C% of 162.5% and CPA with T/C% of 122.0%. Relative resistance was 5.8 for TXL, 3.7 for TXT, 3.4 for DXR, 2.5 for MMC and 2.4 for VDS. MMC, DXR, TXT, TXL and VDS were substrates for MDR1 (18,19), and MDR1-mediated multidrug resistance was also detected even for CD-DST, as well as WST-1 assay.

To ensure higher predictability of clinical efficacy of CD-DST, the exposure concentration and time of anticancer drugs have been defined by the comparative evaluation with clinical results to be one-tenth their maximum clinical concentrations and 24 h, respectively (8,9,25). These concentrations used in three-dimensional CD-DST are about 1% or less of those used in two-dimensional assay (25). In this study, the chemosensitivity of 12 anticancer drugs was evaluated in two-dimensional WST-1 assay at the concentrations used for CD-DST. As shown in Table 1, the values of T/C% in CD-DST were comparable with or higher than the survival fraction (%), being consistent with experimental evidence that cells growing in three-dimensional culture are more resistant to cytotoxic agents than cells in two-dimensional culture (5).

TABLE 1. Chemosensitivity assessed by two-dimensional WST-1 assay and three-dimensional CD-DST

	Concentration (μM)	Survival fraction (%) / WST-1			T/C % / CD-DST		
		HeLa	Hvr100-6		HeLa	Hvr100-6	
5-FU	7.69	48.0±1.7	57.9±2.2	1.2 [a]	66.4±1.0	96.6±9.9	1.5a
SN-38	0.0750	45.5±1.2	44.0±1.7	1.0	38.7±2.2	52.4±1.5	1.4
MMC	0.0897	58.5±1.6	63.9±3.4	1.1	53.1±2.7	135.0±1.8	2.5
CDDP	0.667	77.6±2.4	55.8±4.1	0.7	45.8±2.1	68.5±3.2	1.5
DXR	0.0345	36.9±2.1	89.7±3.4	2.4	47.4±0.7	162.5±21.1	3.4
TXT	0.116	2.3±0.4	51.7±5.5	22.8	8.9±1.0	32.7±1.1	3.7
TXL	0.117	1.9±0.3	85.0±4.4	43.7	13.1±1.1	76.3±4.5	5.8
MTX	0.660	67.0±3.9	88.0±3.2	1.3	100.1±0.4	91.8±5.9	0.9
CPA	0.717	111.2±4.6	99.4±3.6	0.9	108.0±2.2	122.0±1.5	1.1
GEM	1.33	30.6±1.7	32.5±3.1	1.1	19.7±1.2	23.1±0.9	1.2
VDS	0.0117	9.3±1.8	94.3±7.4	10.2	44.2±1.6	104.8±5.5	2.4
VP-16	1.70	39.4±2.0	68.6±3.2	1.7	24.5±1.3	39.5±0.7	1.6

The values were the mean±SD of more than three experiments.
[a] Relative resistance: the ratio of the survival fraction (%) or T/C% for Hvr100-6 cells to that for HeLa cells.

It is noted that the exposure time is 3 days and 1 day for WST-1 assay and CD-DST, respectively. FBS was included in the medium throughout the assay for WST-1 assay, but it was removed with a test anticancer drug on day 2 for CD-DST. These might contribute to tumor growth enhancement or the difference of chemosensitivity. To exclude these possibilities, the WST-1 assay was performed with slight modification of procedure. Table 2 lists the data on the chemosensitivity assessed after 1-day exposure to 12 anticancer drugs in HeLa and Hvr100-6 cells, where a test anticancer drug added on day 1 was removed by exchanging the medium with anticancer drug-free medium on day 2. The exposure time was 1 day as well as that in CD-DST, but the values of survival fraction (%) were similar with those of standard assay procedure as listed in Table 1. Tumor growth enhancement was not observed, and relative resistance was also higher for TXL, TXT, VDS and DXR.

The chemosensitivity was also evaluated under 1-day exposure condition without FBS, i.e., a test anticancer drug and FBS were removed by exchanging the medium with anticancer drug- and FBS-free medium on day 2. Table 3 lists the data on the chemosensitivity. The exposure time was 1 day and no FBS was contained from day 2 to 4, the day of assay, being similar with CD-DST. In this case, the values of survival fraction (%) were comparable with or higher than those of standard assay procedure as listed in Table 1. The culture with serum-free medium results in arrest in G_0/G_1 phase of cell cycle (3), but higher values of survival fraction (%) were not restricted for the anticancer drugs exerting their effects at specific phase of DNA synthesis, including MMC, DXR, TXL and VDS. Tumor growth

enhancement occasionally occurred, but no statistically significant difference was detected. Relative resistance was still higher for TXL, TXT, VDS and DXR. Collectively, three-dimensional culture might contribute to tumor enhancement after exposure to anticancer drugs observed in CD-DST using colorectal adenocarcinoma obtained from Japanese patients.

TABLE 2. Chemosensitivity assessed by two-dimensional WST-1 assay with removing anticancer drugs on day 2

	Concentration (μM)	Survival fraction (%) / WST-1		
		HeLa	Hvr100-6	
5-FU	7.69	61.5±1.2	77.5±3.6	1.3 [a]
SN-38	0.0750	54.7±5.0	60.7±4.5	1.1
MMC	0.0897	66.1±2.5	66.3±3.4	1.0
CDDP	0.667	74.9±3.7	60.4±5.3	0.8
DXR	0.0345	34.9±2.5	88.6±3.9	2.5
TXT	0.116	0.8±0.4	57.8±7.2	68.5
TXL	0.117	0.9±0.9	91.3±4.5	98.7
MTX	0.660	76.7±0.7	88.9±10.4	1.2
CPA	0.717	102.6±4.2	97.9±9.0	1.0
GEM	1.33	28.8±1.5	46.1±2.9	1.6
VDS	0.0117	10.9±0.9	90.2±8.5	8.3
VP-16	1.70	40.9±2.7	71.5±5.7	1.7

The values were the mean±SD of more than three experiments.
[a] Relative resistance: the ratio of the survival fraction (%) for Hvr100-6 cells to that for HeLa cells.

The expression levels of MDR1 and MRP2 were lower in colorectal adenocarcinoma with tumor growth enhancement after exposure to anticancer drugs (15,16). The biopsies were free from the chemotherapy (15,16). However, the deterioration was found in MDR1-overexpression subline, Hvr100-6 cells, and not in parent line, human cervical carcinoma cell line HeLa (Table 1). Hvr100-6 cells were established through stepwise increases of the VLB concentration, and the expression levels of large number of proteins should be altered including MDR1 (6,21). Various anticancer drugs are considered to be the substrate for MDR1, including CPT-11, MMC, anthracyclines, taxans, *vinca*-alkaloids and etoposide (18,19), and multidrug resistance was confirmed in CD-DST as well as WST-1 assay. Further experiments should be conducted to conclude the association with MDR1 and tumor growth enhancement.

TABLE 3. Chemosensitivity assessed by two-dimensional WST-1 assay with removing anticancer drugs and FBS on day 2

	Concentration (μM)	Survival fraction (%) / WST-1		
		HeLa	Hvr100-6	
5-FU	7.69	48.7±6.6	59.9±3.5	1.2 [a]
SN-38	0.0750	56.7±2.7	44.7±2.3	0.8
MMC	0.0897	86.6±9.2	125.6±25.9	1.4
CDDP	0.667	70.3±16.5	60.3±9.0	0.9
DXR	0.0345	62.4±2.7	106.4±7.8	1.7
TXT	0.116	1.3±1.5	101.4±13.6	78.1
TXL	0.117	4.6±0.4	114.4±11.9	24.7
MTX	0.660	99.2±8.7	86.6±10.6	0.9
CPA	0.717	88.8±2.5	81.4±2.7	0.9
GEM	1.33	47.9±1.8	45.4±3.1	0.9
VDS	0.0117	21.1±4.2	119.6±12.1	5.7
VP-16	1.70	54.1±5.5	103.0±9.0	1.9

The values were the mean±SD of more than three experiments.

[a] Relative resistance: the ratio of the survival fraction (%) for Hvr100-6 cells to that for HeLa cells.

CONCLUSION

The values of T/C% in CD-DST were comparable with or higher than those of the survival fraction (%) in WST-1 assay, and modification of WST-1 assay procedure gave similar results, suggesting a higher resistance in three-dimensional than in two-dimensional culture.

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