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The microtiter SDI test is more advantageous than the SDI test for assessing the chemosensitivity of human tumor cells

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The *in vitro* succinate dehydrogenase inhibition (SDI) test was adapted to be used with microtiter plates and this microtiter SDI (mSDI) test was evaluated for clinical use of chemosensitivity testing, as compared to findings with the SDI test. The optimal conditions of the mSDI test were determined: (1) 2–5 × 10^4 cells/well; (2) enzymatic disaggregation of solid tumors with the use of a mixture of 0.2% pronase, 0.25% collagenase, 0.1% DNase for 20 min at 37°C; (3) addition of 10 mM sodium succinate in the colorimetric reaction; and (4) use of dimethyl sulfoxide (DMSO) as a solvent for extraction of formazan product. Good correlations were observed between the mSDI and the SDI tests when S-180 cells ($r = 0.890$–0.996) or 16 human fresh tumor cells ($r = 0.731$–0.999) were exposed to six anti-cancer drugs (carboquone, adriamycin, mitomycin C, aclacinomycin A, cisplatin, 5-fluorouracil). Thus, the mSDI test facilitates testing of a large number of drugs with minimal amounts of specimens, and is expected to replace the SDI test for chemosensitivity testing of clinical tumor cells.

Key words: SDI test, chemosensitivity test, microtiter plate, human tumor cells.

Introduction

Among various chemosensitivity tests, the MTT assay is a rapid, simple and inexpensive method,[1–5] and is currently being used for chemosensitivity testing to screen anti-cancer drugs. The technique also has been adapted for testing fresh leukemic cells from patients.[6–8] Adaptation of this test for specimens of solid tumors has apparently not been reported. The succinate dehydrogenase inhibition (SDI) test shares a common principle with the MTT assay.[1,9] Using the SDI test, we have extensively tested clinical tumor tissues, and obtained interesting findings with respect to gastric, lung, liver, colon, breast, head and neck lesions.[9–13]

To test a large number of drugs with minimal amounts of specimens, such as biopsy specimens, we adapted the SDI test to be used with microtiter plates. We report here our evaluation of the microtiter SDI (mSDI) test, as compared to findings with the SDI test.

Materials and methods

Mouse tumor cells

S-180 cells were maintained by serial intraperitoneal transplantation in 8-week-old male ddY mice. Solid S-180 tumors were derived by subcutaneous injection of 5 × 10^6 cells into the mid-dorsal pelvic region. The tumors were used when they attained a mean diameter of 8 mm.

Human tumor tissues

Solid tumor tissues from 16 Japanese patients (four colon, four thyroid, three gastric, three lung cancers and two malignant lymphomas) were obtained at surgery in our hospital. The excised tissues were immediately immersed in McCoy's 5A solution (Gibco, Grand Island, NY) at 4°C.

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Anti-cancer drugs

The anti-cancer drugs tested were carboquone (CQ), adriamycin (ADM), mitomycin C (MMC), aclacinomycin A (ACR), cisplatin (DDP) and 5-fluorouracil (5-FU). Tumor cells were exposed to various concentrations of the drugs, including the peak plasma and 10-fold the peak plasma concentrations. Sources of these drugs were as follows: CQ was obtained from Sankyo Co., Ltd., Tokyo; ADM, MMC and 5-FU from Kyowa Hakko Co., Ltd., Tokyo; ACR from Sanraku-Ocean Co., Ltd., Tokyo; DDP from Nihon Kayaku Co., Ltd., Tokyo.

SDI test

The SDI test was done as previously described.[9,10] S-180 cells aspirated from the peritoneal cavity were washed three times with McCoy's 5A solution. As these cells were often contaminated with red blood cells, lysis was carried out using a solution of 0.826% ammonium chloride, 0.1% potassium bicarbonate and 0.0037% ethylenediaminetetraacetic acid. Over 95% of the cells were viable, determined using the Trypan Blue dye exclusion method.

Human tumor tissues and solid S-180 tumors were minced with scissors and passed through a No. 32 stainless steel mesh into McCoy's 5A solution containing antibiotics, then washed three times with this solution. The cells were then suspended in minimal essential medium (MEM) (Nissui Seiyaku, Tokyo) with L-glutamine (292 mg/ml), 10% fetal calf serum (Gibco, Grand Island, NY), penicillin (100 μg/ml), streptomycin (100 U/ml) and gentamycin (40 μg/ml), divided into 35 mm plastic dishes, and incubated at 37°C in a humidified 5% CO_2 atmosphere for 3 days in the presence of anti-cancer drugs. After the incubation, the colorimetric reaction was initiated by adding 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl [^{2}H]-tetrazolium bromide (MTT) at a concentration of 0.4 mg/ml and sodium succinate in the range of 0–30 mM. The formazan crystals formed from MMT were dissolved in acetone containing 0.5% trichloroacetic acid, and absorbance at 565 nm was measured with a Beckman DU-50 spectrophotometer. The chemosensitivity was estimated by the percentage of succinate dehydrogenase (SD) activity of the drug-treated cells, compared with that of control cells.

mSDI test

Modifications from the SDI test were as follows. (1) Single cell suspensions were obtained by the combination of mechanical disaggregation and treatment with mixtures of enzymes, including pronase (protease type XXV, Sigma Chemical Co., St. Louis, MO), collagenase (type I, Sigma) and DNase I (type I, Sigma). Tumor tissues were minced with scissors and the resulting fragments were put into a sterile flask containing a mixture of enzymes in McCoy's 5A solution. The enzymatic disaggregation was carried out for 20 min at 37°C with gentle stirring, and terminated by adding sufficient amounts of MEM. The cells were passed through a nylon mesh, washed twice with MEM, pelleted, and re-suspended in MEM. Aliquots (100 μl) of this single cell suspension were dispensed into 96-well microtiter plates (Corning 25860). (2) The formazan crystals were dissolved with 150 μl dimethyl sulfoxide (DMSO), and the absorbance at 540 nm was quantitated with a microtiter plate spectrophotometer (Easy Reader EAR340, SLT Labinstruments, Austria).

Statistical analysis

Correlation coefficient was calculated to describe the strength of the relationship between the SDI and the mSDI tests.

Results and discussion

Cell number vs formazan production

The absorbance of formazan product was proportional to the number of S-180 cells, ranging from 1×10^4 to 1×10^6 cells/ml in the mSDI test (Figure 1a). A single cell suspension of S-180 obtained by tumor disaggregation also showed a linear relationship (Figure 1b). A good correlation between the absorbance and the viable cell number was recognized in the mSDI test. Considering the reliable absorbance read between 0.1 and 1.0 on a spectrophotometer, the optimal cell number was determined to be $2–5 \times 10^5$ cells/ml ($2–5 \times 10^4$ cells/well). This number approximates to optimal numbers reported by other investigators.[3,8,14]

Solvents

The absorption spectra of the formazan product are shown in Figure 2, in that two solvents,

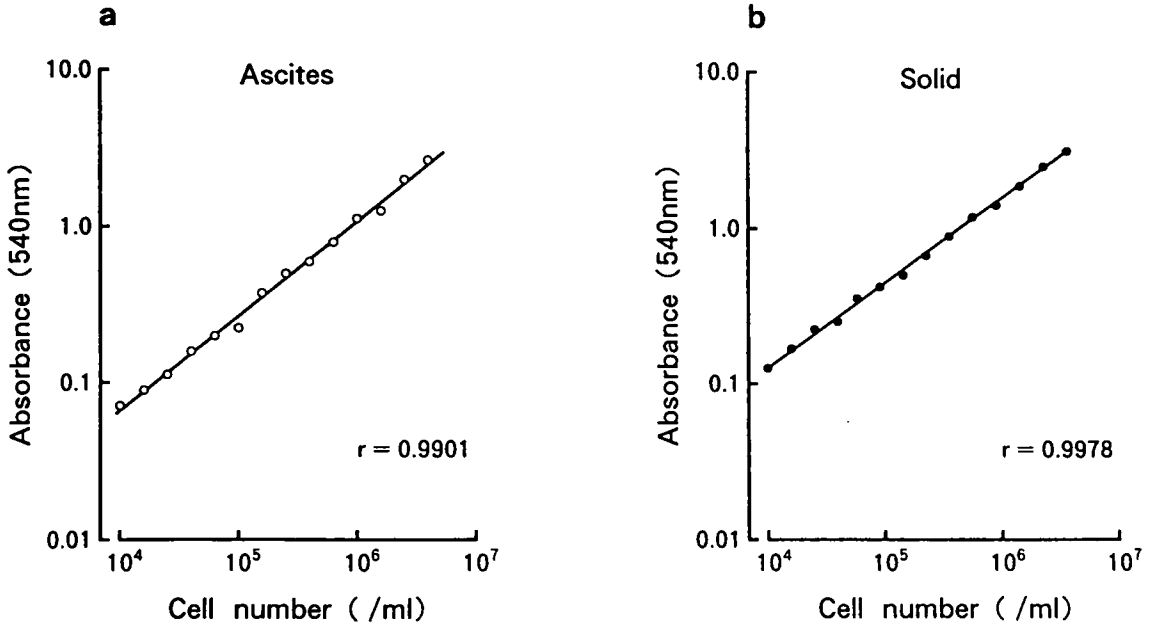

Figure 1. Relationship between the numbers of S-180 cells and the absorbance of formazan product. The tumor cells were collected from ascites (a) or a solid tumor (b) which had been transplanted into the mid-dorsal pelvic region of male ddY mice. Single cell suspension was prepared by tumor disaggregation with a mixture of enzymes, and used for colorimetric reaction. In the colorimetric reaction, the tumor cells were incubated with MTT and sodium succinate for 3 h at 37°C, dissolved in DMSO, and the absorbance at 540 nm was measured.

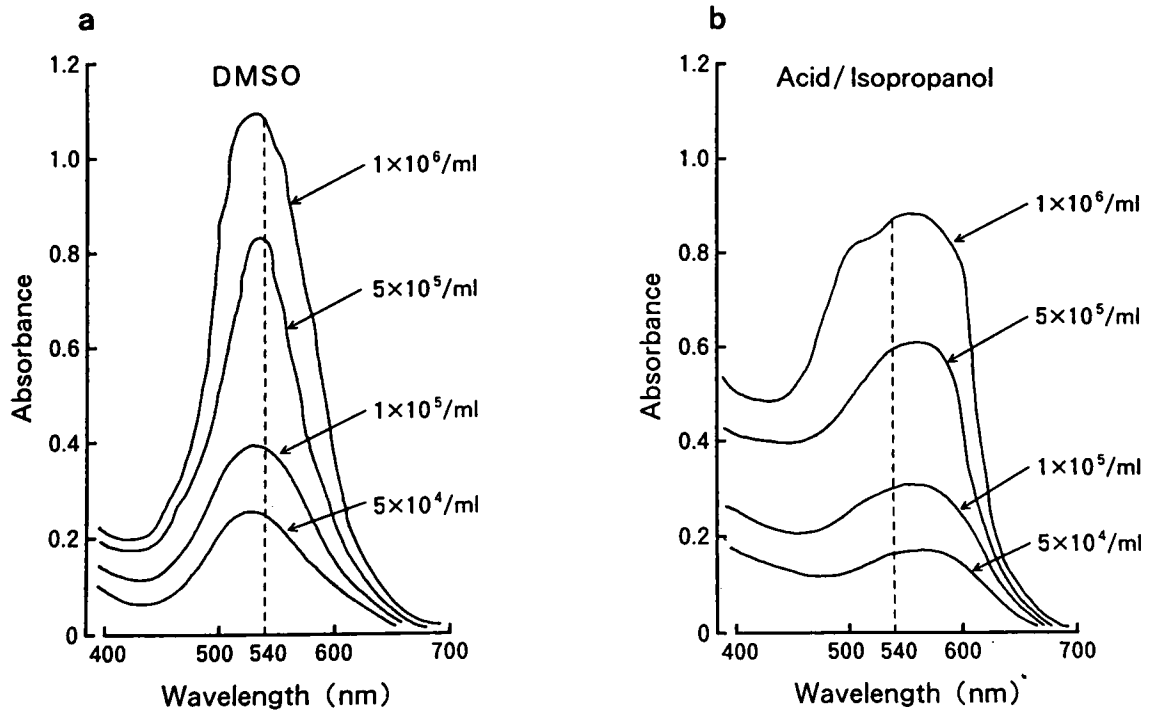

Figure 2. Absorption spectra of formazan, produced by incubation of various numbers of S-180 cells with MTT (0.4 mg/ml) and sodium succinate (10 mM) for 3 h at 37°C, and dissolved in DMSO (a) or acid/isopropanol (b).

acid/isopropanol and DMSO were compared. DMSO gave a higher absorption peak than acid/isopropanol. Acid/isopropanol gave a wide indistinct peak much inferior to that observed using DMSO. A change of cell number between 5×10^3 and 10^5/well led to no shift in the absorption peak, with respect to both solvents. Plumb *et al.*[14] reported a shift according to cell number. Moreover, as DMSO readily dissolved the formazan product even when all the medium was not removed, we used DMSO for the mSDI test, this choice being the same as that by Carmichael[3] and Alley[5], respectively. Acetone was inappropriate for the mSDI test, because it corrodes the material of the microtiter plate.

Effect of incubation time and sodium succinate addition on formazan production

Figure 3a shows the marked increase in absorbance with incubation for up to 3 h, then little further increase. Addition of 10 mM sodium succinate resulted in an increase in absorbance by 1.9-fold. Figure 3b shows the concentration of sodium succinate which most efficiently increased absorbance at 10 mM. In the mSDI test, sodium succinate contributed to increasing the absorbance and the analytical capacity is enhanced.

Comparison in chemosensitivity of S-180 cells assessed by the SDI and the mSDI tests

With respect to change in the SD activity, the mSDI test showed similar behavior to the SDI test when S-180 cells were exposed to various concentrations of each drug (Figure 4). Comparison in the SD activities of pairs at six different drug concentrations showed good correlations between the two tests for each drug ($r = 0.890$–0.996) (Table 1).

Disaggregation of solid tumors

To assay the chemosensitivity of solid tumor cells using the mSDI test, tissue disaggregation to single cells and the collection of viable cells with minimal damage are important factors. We found that the majority of cells in a single cell suspension were not viable when prepared by mechanical disaggregation alone and as determined using the Trypan Blue dye exclusion. We use mixtures of enzymes for disaggregation of solid tumors (Table 2). Two

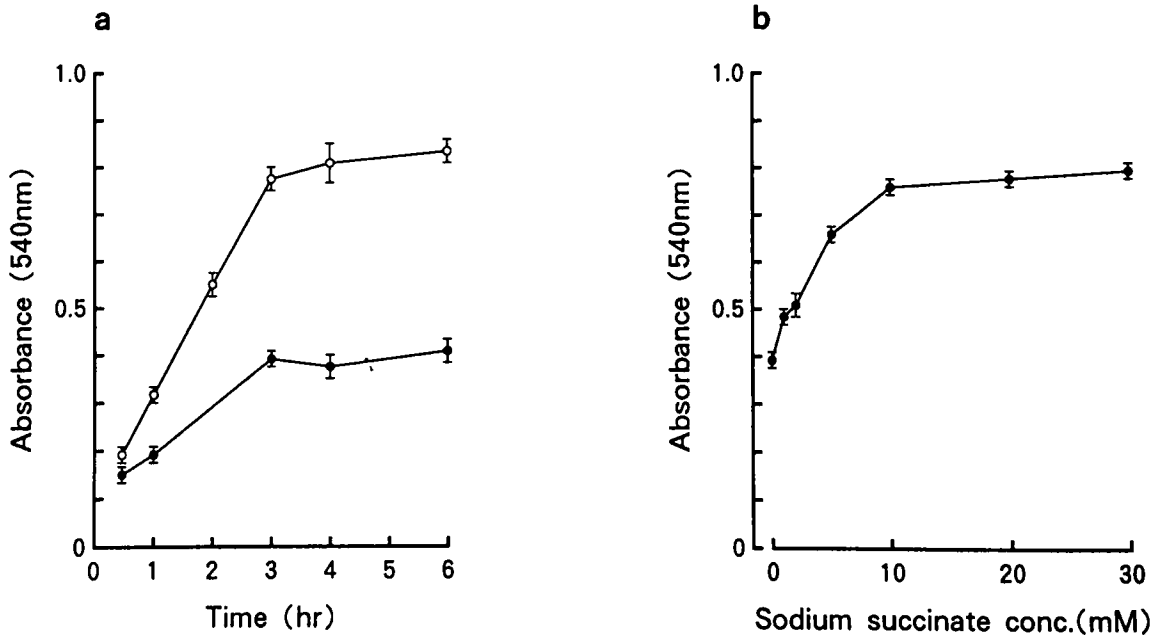

Figure 3. Effect of incubation time and sodium succinate addition on formazan production. (a) S-180 cells (5×10^5/ml) were incubated with MTT (0.4 mg/ml) at 37°C for various times, with (○) or without (●) 10 mM sodium succinate. (b) S-180 cells were incubated with MTT at 37°C for 3 h in the presence of various concentrations of sodium succinate. Each point represents a mean ± standard deviation (bar) of five replicate wells.

mixtures, a pronase-based mixture (A) and a trypsin-based mixture (B) were compared with respect to cell yield and cell viability in the mSDI test. Mixture A showed a higher cell yield and a larger amount of formazan product, indicating a more viable cell population. Pronase was reported to be better than trypsin for the preparation of single cell suspension for colony formation.[15]

The concentration of enzymes for the mSDI test was examined by comparing a high (A) and a low (C) concentration mixture (Table 2). The composition of mixture C was that used by Rasey and Nelson.[15] In an attempt to increase cell yield, we prepared mixture A with a higher enzyme concentration, and this mixture produced a higher cell yield and a larger amount of formazan product.

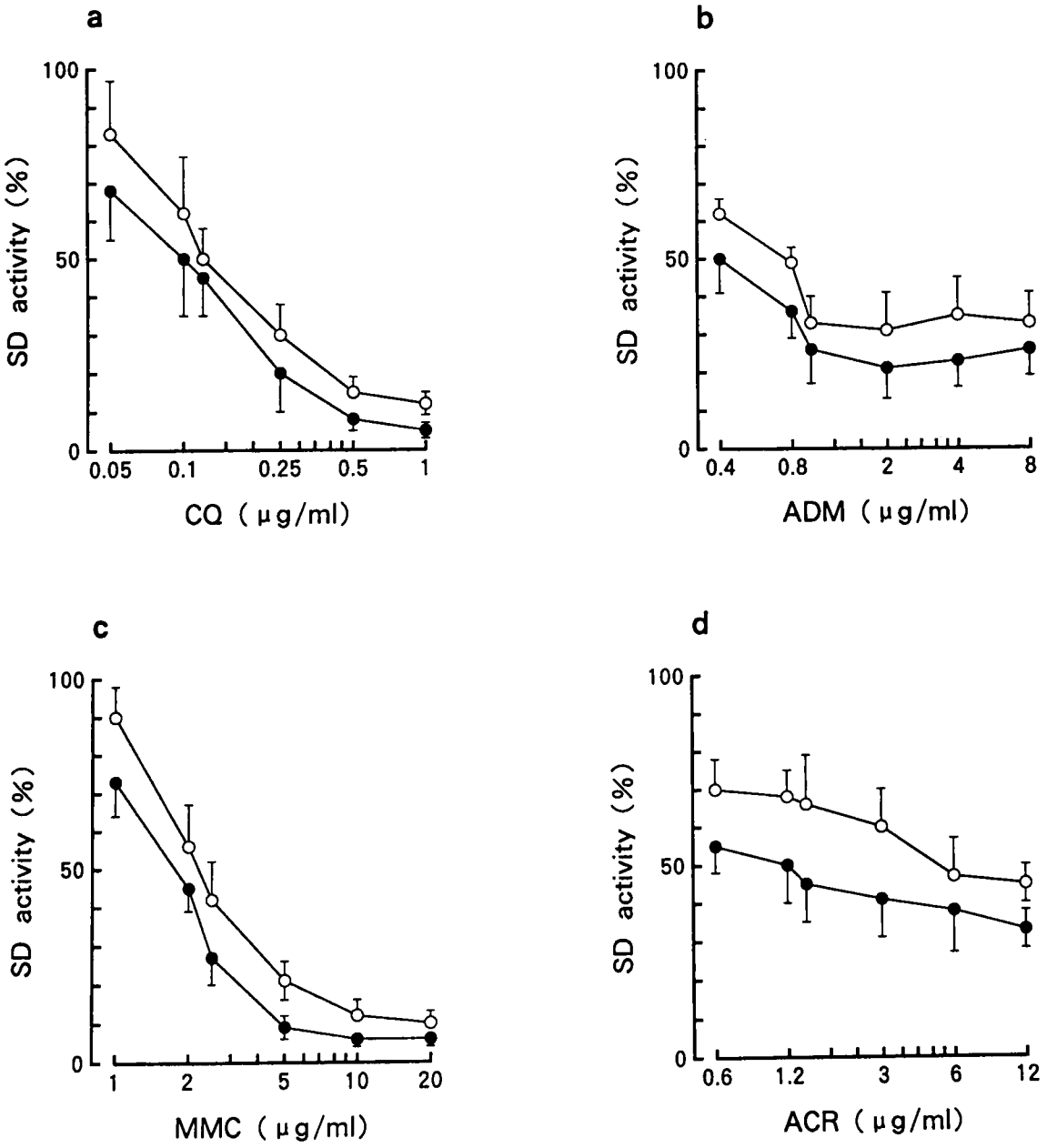

Figure 4. Dose–response curves, expressed as the percentage of SD activity, were assessed by the mSDI (○) and the SDI (●) tests for carboquone (a), adriamycin (b), mitomycin C (c), aclacinomycin A (d), cisplatin (e) and 5-fluorouracil (f) using S-180 cells. The SD activities were measured after continuous drug exposure for 3 days. Each point represents a mean ± standard deviation (bar) of five replicate wells.

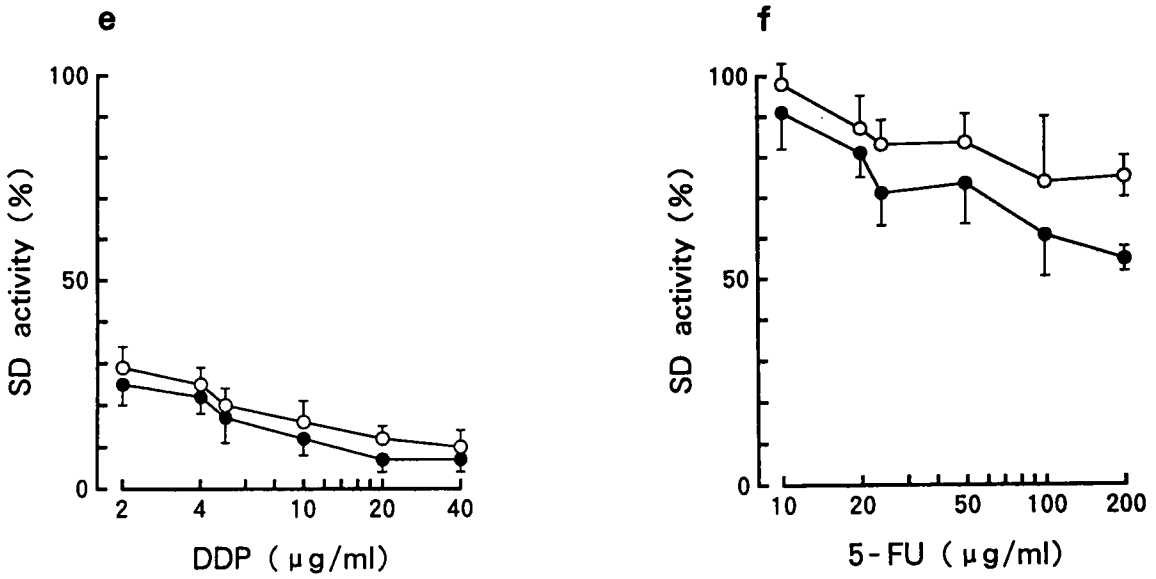

Figure 4 (*Continued*).

Table 1. Correlation between the mSDI and the SDI tests in chemosensitivity of S-180 cells exposed to six anti-cancer drugs

Drug	Drug conc.[a] (μg/ml)	Correlation coefficient[b]
CQ	0.05–1	0.926
ADM	0.4–8	0.979
MMC	1–20	0.996
ACR	0.6–12	0.922
DDP	2–40	0.921
5-FU	10–200	0.890

[a] S-180 cells were exposed to drug at six different concentrations as shown in Figure 4.
[b] Correlation coefficient of the SD activities of pairs at the six different drug concentrations was calculated for each drug.

Comparisons of assays using clinical solid tumors exposed to anti-cancer drugs

Sixteen human samples from colon, thyroid, gastric and lung cancers and malignant lymphomas were tested. Table 3 shows good correlations (r = 0.731–0.999) between the SDI and the mSDI tests in the SD activity of pairs with respect to six anti-cancer drugs. The chemosensitivities to six anti-cancer drugs of these specimens were compared between the two assays. Figure 5 shows the comparisons in the chemosensitivity of four representative cases. A variation in the chemosensi-

Table 2. Disaggregation of S-180 solid tumors with mixtures of enzymes

Enzyme[a]	Mixture		
	A	B	C
Pronase	0.2%	(−)	0.05%
Trypsin	(−)	0.2%	(−)
Collagenase	0.25%	0.25%	0.02%
DNase I	0.1%	0.1%	0.02%
Cell yield[b]	1.9×10^7 cells/g (100%)	1.6×10^7 cells/g (86%)	5.5×10^6 cells/g (29%)
Absorbance[c]	0.74 (100%)	0.43 (58%)	0.57 (77%)

[a] One gram of a solid tumor tissue was disaggregated at 37°C for 20 min.
[b] After disaggregation, viable cells were counted using the Trypan Blue dye exclusion.
[c] Equal amounts of viable cells (2×10^4 cells/well) were used for the colorimetric reaction of the mSDI test.

Table 3. Correlation between the mSDI and the SDI tests in the chemosensitivity of 16 clinical solid tumor cells exposed to six anti-cancer drugs[a]

Case	Type of tumor	Cells plated ($\times 10^4$/well)	Control absorbance[b]		Correlation coefficient[c]
			mSDI	SDI	
1	Colon	5.0	0.339	0.848	0.970
2	Colon	5.1	0.328	0.599	0.999
3	Colon	3.5	0.131	0.113	0.889
4	Colon	4.0	0.364	0.104	0.931
5	Thyroid	3.0	0.407	0.122	0.767
6	Thyroid	5.5	0.804	0.153	0.884
7	Thyroid	3.0	0.692	0.103	0.935
8	Thyroid	2.3	0.122	0.168	0.942
9	Gastric	20.0	0.490	0.165	0.958
10	Gastric	4.0	0.278	0.166	0.960
11	Gastric	9.0	0.261	0.173	0.811
12	Lung	7.0	0.353	0.262	0.971
13	Lung	5.0	0.198	0.447	0.894
14	Lung	10.0	0.299	0.181	0.796
15	Malig. lymphoma	6.4	0.296	0.160	0.731
16	Malig. lymphoma	18.0	0.511	0.445	0.997

[a] Single cell suspensions prepared by enzymatic tumor disaggregation were exposed to CQ, ADM, MMC, ACR, DDP, and 5-FU for 3 days.
[b] The SD activities of control cells (absence of drug) were measured by the mSDI and the SDI tests. The control absorbance above 0.1 was considered to be a valuable assay.
[c] Correlation coefficient of the SD activities of pairs with respect to six drugs was calculated for each case.

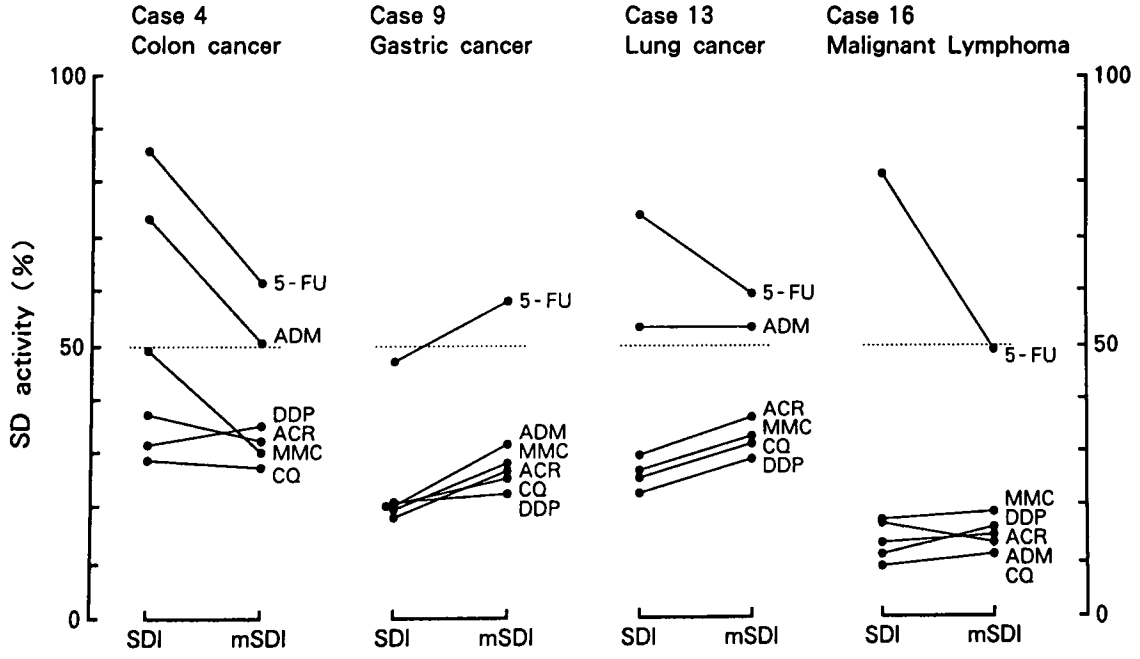

Figure 5. Comparison in the chemosensitivity of four clinical solid tumor cells, assessed by the mSDI and the SDI tests for carboquone (CQ), adriamycin (ADM), mitomycin C (MMC), aclacinomycin A (ACR), cisplatin (DDP) and 5-fluorouracil (5-FU). Single cell suspensions obtained by disaggregation of solid tumors were exposed to a drug for 3 days, and the SD activities were measured by the mSDI and SDI tests.

tivity was noted between patients. The chemosensitivities assessed by the mSDI test were in general agreement with those obtained by the SDI test. There remain a few discrepancies between the two tests when the drug sensitivity in both tests was arbitrarily defined as 50% or a greater reduction of SD activity in the presence of drug, compared to that in the absence of drug. In the clonogenic assay, >50% or >70% inhibition of colony formation was generally defined as sensitive. On the other hand, there are few reports of a correlation between the SDI (or the MTT) test and the results of clinical chemotherapy. Sargent and Taylor[8] reported a correlation between the MTT assay and the clinical results of leukemic patients. They defined >70% inhibition of cell viability as sensitive in the MTT assay, while they assessed complete remission (CR) alone as *in vivo* sensitive. We are examining the correlation between the results of the mSDI test and of clinical chemotherapy to determine the cut-off line in the SD activity for chemosensitivity assessment.

Conclusion

To test a large number of anti-cancer drugs with the minimal amount of specimens in chemosensitivity testing, the *in vitro* SDI test was adapted to be used with microtiter plates and this mSDI test was evaluated for clinical use of chemosensitivity testing, as compared to findings with the SDI test. We found a good correlation between the mSDI and the SDI tests and the optimal conditions for the mSDI test were determined. The mSDI test facilitates testing of a large number of drugs with minimal amounts of specimens. This test is expected to replace the SDI test for chemosensitivity testing of clinical tumor cells.

Acknowledgments

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