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A Rapid Anticancer Drug Screening Assay by [^{14}C] Thymidine Uptake in Cultured Human Cancer Cells

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A rapid screening test by suppression of DNA synthesis in cancer cells was developed with [methyl-^{14}C]-thymidine (^{14}C-TdR), a microculture filtration plate and a radiochromatoscanner. Mitomycin, tamoxifen and 5-fluorouracil (5-FU) were tested against four human gastric cancer cell lines and HeLa cells. The tetrazolium-based colorimetric (MTT) assay underestimated cell inactivation by mitomycin in three cell lines compared with the cell count and the ^{14}C-TdR assays. Inactivation by 5-FU in one cell line by ^{14}C-TdR uptake was considerably lower than that by other methods. Thus neither the radio-labelled DNA precursor uptake nor the MTT assay is suitable for every anticancer drug but they are complementary.
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INTRODUCTION

IN SHORT term assay for the human cancer chemosensitivity test, the tetrazolium-based colorimetric (MTT) assay has been highlighted recently because of using a microculture plate which makes it possible to assay many samples semiautomatically [1–5]. While the estimation of DNA synthesis by cancer cells by radio-labelled DNA precursor uptake is also considered to be useful as well as MTT assay as a cancer chemosensitivity test [6–8], the radio-labelled DNA precursor uptake assay cannot usually be performed semiautomatically.

We have developed a rapid and simple anticancer drug screening test by estimating DNA synthesis in cancer cells using [^{14}C] thymidine (^{14}C-TdR), a microculture filtration plate and a radiochromatoscanner.

Here we compared our ^{14}C-TdR uptake method with the MTT assay and the growth inhibition curve derived by cell count using mitomycin, tamoxifen and 5-fluorouracil (5-FU) against four cultured human gastric cancer cell lines and HeLa cells.

MATERIALS AND METHODS

Four human gastric cancer cell lines and HeLa cells were used. Three gastric cancer cell lines, MKN45, MKN74 and KATOIII, originating in the School of Medicine, Niigata University [9] and the NEDATE cell line was established in our laboratory from a patient with advanced gastric cancer of undifferentiated type. These cells were grown in RPMI-1640 medium (Nissui Co, Tokyo) containing 10% fetal calf serum (Flow) in a CO_2 incubator at 37°C.

Tamoxifen (ICI) was dissolved initially in 50% methanol and diluted with the culture medium and other drugs were diluted directly with the culture medium. Mitomycin (Kyowa Hakkou Kogyo, Tokyo) was used in the range of 0.1–1 μg/ml; tamoxifen, 1.4–11.2 μg/ml and 5-FU (Kyowa Hakkou Kogyo), 0.1–10 μg/ml.

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Equal numbers of single cells (8–10 × 10^3 cells) in exponentially growing phase in 100 μl of culture medium were seeded into the 96 wells of a culture plate [Microwell F96 plate, (Nunc) for MTT assay and Millititer-GV filtration plate (Millipore) for ^{14}C-TdR uptake assay]. The Millititer-GV filtration plate consists of a microporous membrane filter (pore size 0.2 μm) sealed to the underside of a polystyrene plate. After 3 h of initial culture, 100 μl of the various concentrations of the drugs were added to each well and the cells were exposed to the drugs in a CO_2 incubator at 37°C for 16, 40 or 65 h.

^{14}C-*TdR uptake assay*

After treatment with the drugs, 3 kBq of ^{14}C-TdR (N.E.N.: s.p. 1.9 TBq/mmol) per 10 μl was added to each well of the plate and the cells were labelled by incubation for 3 h in a CO_2 incubator at 37°C, the cell suspension in the wells was removed with a vacuum/pressure pump, 200 μl of cold 10% trichloroacetic acid was added to each well, and the suspension was aspirated again after 1 h. The plate membrane where the ^{14}C-TdR incorporated cells remained was washed with 200 μl of cold Dulbecco's phosphate buffered saline (PBS) twice, dried and scanned for 30 min with a radiochromatoscanner (AMBIS, Radioanalytic Imaging System, San Diego). To re-evaluate the data obtained by the radiochromatoscanner, the microporous membrane filter paper was detached from the underside of the plate and cut into 96 pieces. Then the ^{14}C-activity of each cut sample was counted for 10 min with a Beckman LS9800 liquid scintillation counter.

MTT assay was performed by the method of Mossman [1] with minor modification and cell count assay by our published procedure [10].

In all assays, the cell inactivation ratio by the drug was expressed as the percentage of the value of non-treated cells.

In data processing, the mean and standard deviation were obtained from four tubes for cell count, six wells for the MTT assay and four wells for the ^{14}C-TdR uptake assays.

RESULTS

To evaluate the results obtained by the assays examined, the mean IC_{50} values with 90% of confidence intervals were determined from the cell inactivation rate curve obtained by

Table 1. IC_{50} values for cultured human cancer cell lines exposed to mitomycin, tamoxifen and 5-FU by three individual assays

Cell line	Exposure time (h)	Cell count	MTT	^{14}C-TdR uptake	
				L.S.C.*	R.C.S.†
Tamoxifen					
NEDATE	16	11.26 (0.91)	2.82 (0.45)	5.63 (0.66)	n.d.‡
	40	5.63 (0.78)	3.49 (0.35)	9.01 (0.54)	11.20 (1.00)
	65	3.49 (0.62)	2.98 (0.23)	6.19 (0.62)	5.63 (0.67)
Kato-III	16	11.26 (0.88)	13.73 (0.61)	11.26 (0.89)	11.26 (0.94)
	40	5.29 (0.70)	7.32 (0.83)	7.88 (0.91)	7.88 (0.48)
	65	2.92 (0.57)	4.05 (0.87)	2.70 (0.18)	3.04 (0.32)
MKN45	16	20.83 (1.93)	22.52 (1.76)	n.d.	n.d.
	40	7.21 (0.80)	7.09 (0.56)	8.56 (0.74)	9.01 (0.95)
	65	4.84 (0.33)	5.18 (0.36)	4.67 (0.51)	4.67 (0.08)
MKN74	16	17.68 (1.41)	9.01 (0.37)	n.d.	n.d.
	40	7.99 (0.25)	5.40 (0.47)	8.11 (0.80)	9.01 (1.23)
	65	3.10 (0.58)	4.22 (0.31)	1.97 (0.31)	2.31 (0.28)
HeLa	16	n.d.	n.d.	n.d.	n.d.
	40	9.34 (0.78)	8.44 (0.33)	11.26 (0.78)	11.03 (0.93)
	65	5.63 (0.91)	7.66 (2.55)	6.87 (0.40)	5.80 (0.45)
Mitomycin					
NEDATE	16	0.10 (0.02)	1.20 (0.12)	0.08 (0.005)	0.10 (0.01)
	40	0.08 (0.01)	0.42 (0.07)	0.05 (0.007)	0.06 (0.01)
	65	0.03 (0.006)	0.07 (0.01)	0.03 (0.007)	0.04 (0.01)
Kato-III	16	1.32 (0.25)	n.d.	0.80 (0.06)	1.20 (0.08)
	40	0.44 (0.09)	n.d.	0.20 (0.02)	n.d.
	65	0.07 (0.02)	0.30 (0.02)	0.06 (0.008)	0.05 (0.02)
MKN45	16	n.d.	n.d.	n.d.	n.d.
	40	0.08 (0.01)	0.43 (0.03)	0.08 (0.01)	0.08 (0.01)
	65	0.05 (0.01)	0.08 (0.02)	0.02 (0.005)	0.03 (0.005)
MKN74	16	1.16 (0.14)	n.d.	0.59 (0.04)	0.58 (0.06)
	40	0.08 (0.02)	2.50 (0.23)	0.06 (0.005)	0.07 (0.02)
	65	0.04 (0.01)	0.84 (0.07)	0.03 (0.008)	0.03 (0.01)
HeLa	16	0.30 (0.05)	n.d.	n.d.	n.d.
	40	0.09 (0.01)	2.48 (0.11)	0.07 (0.009)	0.06 (0.02)
	65	0.06 (0.02)	0.68 (0.02)	0.04 (0.01)	n.d.
5-FU					
NEDATE	16	1.00 (0.12)	3.2 (0.26)	n.d.	n.d.
	40	0.30 (0.04)	1.5 (0.08)	10.00 (0.37)	10.00 (0.88)
	65	0.15 (0.03)	0.8 (0.05)	6.00 (0.21)	6.00 (0.72)
Kato-III	16	n.d.	11.5 (0.86)	6.50 (0.40)	6.00 (0.69)
	40	0.40 (0.05)	1.6 (0.09)	0.15 (0.02)	0.15 (0.03)
	65	0.40 (0.07)	1.0 (0.12)	0.25 (0.03)	0.25 (0.11)
MKN45	16	6.00 (0.22)	5.50 (0.43)	10.00 (0.37)	9.50 (0.52)
	40	0.30 (0.04)	0.30 (0.03)	0.40 (0.08)	0.40 (0.10)
	65	0.50 (0.03)	0.40 (0.03)	0.45 (0.03)	0.45 (0.07)
MKN74	16	0.40 (0.03)	n.d.	11.00 (0.35)	12.00 (0.74)
	40	0.30 (0.05)	3.50 (0.32)	4.25 (0.16)	4.30 (0.19)
	65	0.20 (0.02)	0.70 (0.06)	0.25 (0.04)	0.25 (0.08)

* L.S.C. = Liquid scintillation counter. † R.C.S. = Radio chromatoscanner. ‡ n.d. = Not determined. Values in parenthesis are 1 S.D.

each method and are shown in Table 1. The IC_{50} was defined as the concentration of the drug which produced 50% reduction of viable cells for the growth inhibition curve, the absorbance at 540 nm for MTT assay or the ^{14}C activity for the ^{14}C-TdR uptake assay. The range of coefficients of variation (CV) of within assays were 5.1–36.6% in the cell count, 3.9–33.2% in the MTT assay, 3.5–20.0% in the ^{14}C-TdR uptake assay by liquid scintillation counter and 1.7–44.0% in the ^{14}C-TdR uptake assay by radiochromatoscanner. The lower was a percentage of the survival, the higher CV was obtained up to 33% around the back ground levels. The range of the inter assay variance of three methods in three experiments were as follows: 2.4–8.3% by MTT assay, 5.2–18.4% by cell count assay, 7.8–21.0% by ^{14}C-TdR uptake assay with liquid scintillation counter and 8.9–23.1% by ^{14}C-TdR uptake assay with radiochromatoscanner.

The ^{14}C-TdR uptake determined with the radiochromatoscanner was almost identical with that determined with the liquid scintillation counter regardless of cell line, exposure time and drug as shown in Table 1.

All assays showed approximately the same IC_{50} values by tamoxifen (Table 1). No cytotoxicity was found in the control, which contained 1% of methanol in all assays (data not shown).

After exposure to mitomycin, the cell count and the ^{14}C-TdR uptake assays showed similar results with all the cell lines, but the IC_{50} values in MKN74 and HeLa cells obtained by the MTT assay were less than one-tenth of those obtained by the cell count or the ^{14}C-TdR uptake assay (Table 1).

When 5-FU was used, all assays yielded similar cell inactivation rates in MKN45. But, particularly, in NEDATE cell line exposed for 40 or 65 h, the IC_{50} values obtained by the MTT assay and ^{14}C-TdR uptake assay were one-fifth and less than one-thirtieth of those by the cell count assay, respectively (Table 1).

DISCUSSION

Considering the assay's cost and the radiotoxicity of ^{14}C, the optimal quantity of ^{14}C-TdR used for one well and the labelling time were determined as below 3.7 kBq and 3 h, respectively (data not shown). This condition yielded sufficient incorporation to estimate the inactivation rate with the radiochromatoscanner in which the counts ranged from 20 to 4000 cpm (background: 20 cpm). The efficiency of counting ^{14}C with the radiochromatoscanner was 25%, lower than the 90% with the liquid scintillation counter. However, the results obtained by both counting methods were almost identical to each other as shown in Table 1. A radiochromatoscanner needs only 30 min for counting 96 holes while the liquid scintillation method requires punching out the filter membrane, putting the pieces in scintillation vials and overnight counting of their radioactivity. ^{3}H-TdR was not used because the counting efficiency of ^{3}H by the radiochromatoscanner was too low (0.2%).

When four cell lines (NEDATE, KATOIII, MKN74 and HeLa cells) were exposed to mitomycin, the IC_{50} determined with the MTT assay were significantly higher than those determined by cell count and ^{14}C-TdR uptake assays (Table 1). We observed that the size of these cells exposed to mitomycin became larger than that of untreated cells. Jabbar *et al.* observed that the MTT assay underestimated the growth inhibitory effects of interferons in a human lung cancer cell line; the cells treated with interferon showed an increase in size accompanied with increased mitochondrial activity, which might contribute to increased formation of formazan converted from the MTT reagent [11]. Our findings agreed with theirs in the underestimated effect of mitomycin by the MTT assay and the observation of the increased cell size. Another cause of the increase in formazan formation was considered to be as follows: mitomycin changes the cell membrane permeability, in consequence, the MTT reagent passively enters into the cells and enhance formazan formation. However, there is not yet any evidence for this hypothesis, our findings indicate that mode of action of the drug against the target cell including possible alteration of membrane permeability should be considered to obtain the correct results with the MTT assay.

The significant underestimation of the rates of cell inactivation by 5-FU measured with the ^{14}C-TdR uptake assay occurred with NEDATE cells at every exposure time and MKN74 cells exposed for 16 and 40 h (Table 1). The ^{3}H-TdR incorporation assay is reported to underestimate the inactivation by 5-FU and methotrexate in comparison with the human tumour clonogenic assay [6], because antimetabolites of a DNA precursor block *de novo* thymidine synthesis, leading to the reduction of the intracellular thymidine pool and thereby the utilisation of exogenous thymidine is enhanced [6, 12]. Besides, Boudewijn *et al.* reported that various derivatives of 5-fluoro-pyrimidine strongly inhibit the incorporation of tritiated deoxyuridine into DNA of human tumor cells originated from colon carcinoma or head and neck cancer, which reflects the inhibition of thymidilate synthase [13]. These findings and our results show that a DNA precursor uptake assay is not satisfactory for screening for antimetabolites of DNA precursors.

From our experiments with five cancer cell lines in three individual assay methods, neither the radio-labelled DNA precursor uptake nor the MTT assay is suitable for every anticancer drug but they are complementary. Therefore, the precise activity of anticancer drugs must be determined with these two tests according to their different mechanisms.

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Prediction of Doxorubicin Resistance in Gastrointestinal Cancer by P-Glycoprotein Staining

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Feasibility of immunohistochemical staining of P-glycoprotein for the prediction of doxorubicin resistance in gastrointestinal cancers was examined. Among 10 cancer cell lines which consist of two gastric cancer cell lines and eight colon cancer cell lines, seven cell lines were stained positively by the monoclonal antibody to P-glycoprotein, C219. In consequence of the evaluation on the effect of doxorubicin on these tumour cells by means of succinic dehydrogenase inhibition test (SDI test), zero out of seven cell lines stained positively by C219 was sensitive to doxorubicin, but two out of three cell lines stained negatively were sensitive. Among 23 fresh surgical specimens of gastrointestinal cancers which consisted of 15 gastric cancers and eight colon cancers, seven tumour tissues were stained positively by C219. All P-glycoprotein positive tumours were resistant to doxorubicin. On the other hand, four of 16 P-glycoprotein negative tumours were sensitive to doxorubicin. These data indicate that positively stained cancer cells by C219 are resistant to doxorubicin.
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INTRODUCTION

FOR THE CHEMOTHERAPY of gastrointestinal cancers, doxorubicin is one of the most effective drugs. Combination chemotherapy regimens including doxorubicin have been used on patients with gastrointestinal cancers, although the response rates are not satisfactory and complete responses are generally uncommon. Recently, it has become clear that a 170 kD membrane glycoprotein (P-glycoprotein) encoded by the MDR1 gene functions as an energy-dependent drug efflux pump of specified anticancer drugs such as doxorubicin and vincristine [1]. When various molecular and immunologic techniques were used, P-glycoprotein expression was detected in human gastric and colorectal carcinoma cell lines and surgical specimens from patients with gastrointestinal cancers [2–5]. From these basic findings, P-glycoprotein is thought to play an important role in the doxorubicin resistance of patients with gastrointestinal cancers who have not been treated by doxorubicin before the surgery. In this study, we compared the *in vitro* sensitivity patterns of cytotoxic drugs and P-glycoprotein expression by immunohistochemical staining in cell lines and fresh specimens of gastrointestinal carcinomas.

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MATERIALS AND METHODS

Cell lines and clinical samples

Human gastric cancer cell lines that are now well established in serial subculture were used. MKN-28 and AZ521 were provided by Japanese Cancer Research Resources Bank. Human colorectal carcinoma cell lines, SW1083, SW1116 and SW1222 were presented by Sloan Kettering Cancer Research Institute and LoVo, Colo201, DLD-1, CaR-1 and SW-837 were provided by Japanese Cancer Research Resources Bank. Human epidermoid carcinoma cell line, KB and its colchicine resistant cell line KB^{chr} were donated by Dr Kuwano, Ohita Medical University. Clinical samples were obtained by surgical specimen in our department of surgery. Specimens were resected from the patients with stomach and colon cancer.