Eur J Cancer Clin Oncol, Vol. 24, No. 9, pp. 1421–1424, 1988.
Printed in Great Britain

0277–5379/88$3.00 + 0.00

A Simplified Tritiated Thymidine Incorporation Assay for Chemosensitivity Testing of Human Tumors

TETSUYA WADA,* TSUYOSHI AKIYOSHI* and YASUYA NAKAMURA†

*Department of Surgery, Medical Institute of Bioregulation, Kyushu University, Beppu 874, Japan and †Department of Surgery, Oita Prefectural Hospital, Oita 870, Japan

Abstract—*Chemosensitivity testing was performed using a simplified rapid assay based on tritiated thymidine incorporation. In this assay,* 1.5 × 10^5 *tumor cells were cultured for* 72 h *in double-layer agarose in* 25 mm *plastic dishes, which had a Millipore membrane sealed to the underside. After* 24 h *labeling of the cells with tritiated thymidine, cells were collected on the membrane by evacuating the solubilized medium through the filter sealed to the underside of the dish, which was placed in a boiling water bath. The radioactivity of the collected cells was counted by liquid scintillation. We found that* 65% *of* 68 *malignant tumors gave evaluable chemosensitivity results. Overall, chemotherapeutic drugs caused a* 38% *response rate* in vitro. *Twenty instances were evaluable for* in vitro–in vivo *correlations. The predictive accuracies were* 93% *for resistance and* 67% *for sensitivity. The results indicate that, with technical simplicity and shorter time course* (4 *days*), *this assay may be useful for chemosensitivity testing in human tumors.*

INTRODUCTION

TECHNIQUES for measuring tritiated thymidine incorporation by tumor cells plated in soft agar have been reported and, based on these methods, a rapid assay for *in vitro* chemosensitivity testing has been developed [1–3]. Further, the assay technology was modified with a miniaturized version (MINI-assay), improving the evaluability rates, precision, and reliability of chemosensitivity results [4].

Previously, we have developed a simplified method for determining tritiated thymidine incorporation into tumor cells in semisolid medium [5]. The present study was undertaken to investigate the applicability of our assay system to the *in vitro* chemosensitivity testing of human malignant tumors.

MATERIALS AND METHODS

Single cell suspensions from tumor tissue were prepared by enzymatic disaggregation, as described by Slocum *et al.* [6], with minor modifications. Briefly, solid tumor specimens were trimmed free of normal and necrotic elements and were minced with a scalpel. The minced tissue was incubated in mixture containing 0.8% collagenase and 0.002% DNase in RPMI 1640 medium with 10% fetal bovine serum at 37°C for 30 min. Cells freed from the tissue were collected by filtering the minced tissue through nylon gauze and by washing with fresh medium. Malignant effusions were collected in heparinized bags (100 U/ml). After centrifugation for 10 min, the cells were washed twice with the same medium.

Cells were cultured in plastic dishes 25 mm in diameter to which a Millipore membrane, pore size of 5 μ, was sealed on the underside (Millicell, Millipore Corporation, U.S.A.). This dish contained two layers of agarose: a feeder and an upper layer. The feeder layer consisted of double-strength Eagle's minimal essential medium (MEM), 15% fetal bovine serum and 0.6% agarose. 1.5 ml of the solution was dispersed into each dish. The upper layer in which the tumor cells were suspended consisted of double-strength MEM supplemented with 15% fetal bovine serum and 0.3% agarose. 0.5 ml of the mixture was pipetted on the feeder layer. The final concentration of cells in each culture was 1.5 × 10^5 viable cells in 0.5 ml of agarose medium.

Accepted 27 April 1988.
Correspondence should be addressed to: Tsuyoshi Akiyoshi, M.D., Department of Surgery, Medical Institute of Bioregulation, Kyushu University 69, Beppu 874, Japan.

If drugs were to be added, they were added in suspension in 20 μl phosphate buffered saline (PBS) over the top layer. Drugs used, and their final concentration in agarose, were adriamycin, 0.4 μg/ml; mitomycin C, 3.0 μg/ml; 5-fluorouracil, 10.0 μg/ml; *cis*-platinum, 1.0 μg/ml; bleomycin, 2.0 μg/ml; l-phenylalanine mustard, 5.0 μg/ml; vincristine, 0.5 μg/ml; ACNU, 20.0 μg/ml; and carboquone, 0.03 μg/ml. A positive control was included by adding 20 μl of mercuric chloride in PBS (final concentration, 100 μg/ml) to each of three dishes. The dishes were placed in a humidified incubator at 37°C in the presence of 5% CO_2.

After 72 h of incubation, each culture was labelled with 5 μCi tritiated thymidine, and the dishes were again incubated for 24 h. To completely solubilize the agarose, the dishes were placed in a boiling water bath connected to assembled vacuum holders. The solubilized medium was evacuated through membrane filters sealed on the underside of the dish. Therefore, cells in the medium were collected on the membrane. After several washings with boiled PBS, the dishes were punched out. The radioactivity of each disc was determined by counting in a liquid scintillation counter. In order to compare the results of this assay with those of the previous method [1–4], incorporation of thymidine was terminated by transfering the agarose layer of each dish to a centrifuge tube and boiling the tubes for 15 min in a water bath. The volume was brought up to 13 ml with PBS and the tubes were centrifuged. The pellets were washed twice with PBS before being dissolved in 3 ml of 0.86 N KOH at 80°C for 1 h. Tubes were cooled to below 4°C, and the hydrolysates were precipitated by addition of 30 μl of 1% human serum albumin and 2.4 ml of ice-cold 30% trichloroacetic acid (TCA). After overnight storage at 4°C, precipitates were collected by centrifugation. The pellets were washed with 5% TCA, dissolved in 0.3 ml of 0.075 N KOH, and transferred to a scintillation vial for counting of the radioactivity.

An assay was considered evaluable if the average count of the untreated (no drug) controls was greater than 300 dpm, if the positive control gave at least 80% inhibition of thymidine uptake, and if the coefficient of variation for the controls was less than 50 [4]. *In vitro* sensitivity to a chemotherapeutic drug was defined as greater than 80% inhibition of tritiated thymidine incorporation in drug-treated dishes compared to untreated controls. Clinical response to chemotherapeutic drugs was assessed by review of patient records. A complete response was defined as the disappearance of all clinical manifestations of disease for longer than 1 month. A partial response was defined as at least a 50% reduction in the size of all measurable disease for at least 1 month. Only complete or partial responses were considered responses *in vivo*.

RESULTS

The relationship between tritiated thymidine uptake by the same tumor as measured by the assay using the precipitation procedure and by this simplified assay was determined in nine different tumors. As shown in Table 1, there was significant correlation between the dpm values in these two assays ($r = 0.971$, $P < 0.01$).

Sixty-eight tumor specimens were obtained from patients with various malignant tumors, including 20 with gastric carcinoma, 19 with colon carcinoma, 18 with breast carcinoma, three with ovarian carcinoma, three with pancreatic carcinoma, two with lung carcinoma, two with esophageal carcinoma and one with sarcoma. Overall, 65% of the tumor fulfilled the criteria for *in vitro* growth (≥300 dpm thymidine incorporation, ≥80% inhibition of incorporation by the positive control, and <50 coefficient of variation for the controls) (Table 2). Tumor types included carcinoma of the stomach, of which 12 of 20 were evaluable (60%), colon, 12 of 19 (63%); breast, 12 of 18 (67%); and other, eight of 11 (73%).

In vitro activity of chemotherapeutic drugs was assessed using the cutoff level of 80% inhibition of thymidine incorporation (Table 3). A total of 191 tests were performed in 44 patient tumors which had sufficient growth (average, 4.3 drugs/patient).

Table 1. Relationship between tritiated thymidine incorporation as measured by the assay using the precipitation procedure and the simplified assay

Patient No.	Tumor type	Precipitation procedure (dpm)	Simplified assay (dpm)
1	Gastric	122 ± 31	65 ± 12
2	Colon	2231 ± 312	2504 ± 482
3	Colon	338 ± 66	416 ± 20
4	Colon	59 ± 4	48 ± 5
5	Breast	574 ± 119	570 ± 155
6	Breast	393 ± 60	387 ± 108
7	Ovarian	5524 ± 530	4777 ± 721
8	Esophageal	342 ± 97	709 ± 125
9	Sarcoma	1767 ± 208	1547 ± 292

Table 2. In vitro *growth rate by tumor type*

Tumor type	No. plated	No. with growth	Successful (%)
Gastric	20	12	60
Colon	19	12	63
Breast	18	12	67
Ovarian	3	3	100
Pancreatic	3	2	66
Lung	2	1	50
Esophageal	2	1	50
Sarcoma	1	1	100

Table 3. *Number of responses* in vitro (*at least* 80% *inhibition*)

Agent	Gastric	Colon	Breast	Other	Total (%)
Adriamycin	2/9	3/12	2/9	1/7	8/37 (22)
Mitomycin C	4/8	5/11	2/6	1/7	12/32 (38)
5-Fluorouracil	4/9	4/9	4/7	2/7	14/32 (44)
Cis-platinum	5/9	2/6	4/7	2/4	13/26 (50)
Bleomycin	2/7	2/4	3/7	1/3	8/21 (38)
L-PAM	6/8	0/5	1/4	1/5	8/22 (36)
Vincristine	1/1	1/2	2/4	—	4/7 (57)
ACNU	1/1	1/2	1/2	0/2	3/7 (43)
Carboquone	1/2	0/1	1/2	0/2	2/7 (29)
Total	26/54	18/52	20/48	8/37	72/191
(%)	(48)	(35)	(42)	(22)	(38)

All of the drugs tested were found to have some *in vitro* activity (22–57% specimens sensitive). Overall, these drugs caused a 38% response rate *in vitro*. There was no significant difference in relative frequencies of *in vitro* sensitivity by histologic types of malignant tumors.

Correlations between *in vitro* inhibition of thymidine incorporation and clinical response were available in 20 instances (Table 4). In six instances in which the tumor was found to be sensitive to a particular drug *in vitro* (at least 80% inhibition of thymidine incorporation), there were four clinical responses observed (67% prediction accuracy for sensitivity). In 14 instances where the tumor was resistant *in vitro* (less than 80% inhibition of thymidine incorporation), there was only one clinical response (93% prediction accuracy for resistance).

Table 4. *Correlation between* in vitro *and* in vivo *response to chemotherapy*

Sens/Sens	Sens/Res	Res/Sens	Res/Res
4	2	1	13

Prediction accuracy for sensitivity = 4/6 (67%).
Prediction accuracy for resistance = 13/14 (93%).
Sens: sensitive; Res: resistant.

DISCUSSION

A rapid assay for *in vitro* chemosensitivity testing measuring tritiated thymidine incorporation has been described by Tanigawa *et al.* [1]. Their extended study demonstrated that the rapid assay offered several advantages over the soft agar clonogenic assay, including higher success rate, avoidance of clumping artifacts, short time course (5 days), and low false-negative rate [2]. Previously, we developed a simple method for measuring tritiated thymidine incorporation by cells from tumor tissue plated in semisolid medium [5]. This method is technically much simpler than their thymidine incorporation assay, as use was made of a Millipore membrane. In their assay system, thymidine incorporation was measured after overnight precipitation of the labeled cell pellet obtained by centrifugation of the solubilized agar layer containing tumor cells. In our method, however, the labeled cells could be readily collected on the membrane by evacuating the solubilized medium through membrane filters sealed on the underside of the dish. Therefore, our assay yields results earlier (4 days), since the precipitation procedure was unnecessary.

In their study, at least 97% of the labeled cells with tritiated thymidine in the soft agar culture were shown to be malignant using autoradiographic examination. Then there was a strong correlation between tritium incorporation measured by autoradiographs and scintillation counting of the precipitated materials [1]. In this method, we simply collected the labeled cells on the membrane without the precipitation procedure and measured the tritiated thymidine incorporation. There was a significant correlation between the dpm values in this simplified assay and the assay using the precipitation procedure.

In their thymidine incorporation assay, 1.0 ml of tumor cell suspension was dispersed to 35 mm wells of a 6-well plate over the feeder layer of agar, yielding 4×10^5 cells/well [1–3]. Further, they developed a modified version of this assay system (MINI-assay) [4], in which agarose was substituted for agar and 1.5×10^5 tumor cells were plated in double-layer agarose in 16 mm wells of a cluster dish. In our assay system, 1.5×10^5 viable cells in 0.5 ml agarose medium were pipetted on the feeder layer in plastic dishes 25 mm in diameter. In the thymidine incorporation assay, Sondak *et al.* [3] reported that 59% of 819 tumor specimens fulfilled the criteria for *in vitro* growth. However, breast and colon carcinoma grew poorly in their assay (41% and 47%, respectively). Then they performed the MINI-assay with a total of 351 tumors and evaluable chemosensitivity assays were obtained on 280 (80%) of these [4]. The success rates for breast and colon carcinoma were 63% and 68%, respectively. In our study, 68 tumors were processed, with an overall success rate of 65%. Tumors from breast

and colon exceeded 60% evaluability, which was almost the same level as that in MINI-assay.

With the 80% cutoff for sensitivity, most drugs were found to have lesser *in vitro* activity than reported previously in the thymidine incorporation assay, testing the same agents at the same concentrations (37–72% of specimens sensitive) [3]. That could be attributable to the difference in the tumor types tested, since our study mainly included colon, breast and gastric carcinoma, which had been shown to have relatively lower *in vitro* sensitivity [7].

The rapid assay based on tritiated thymidine incorporation was shown to be particularly accurate for predicting clinical resistance to chemotherapy, whereas there was an overprediction for sensitivity [2–4]. While based on much smaller numbers, our overall predictive accuracies were 93% for resistance and 67% for sensitivity. Since the higher false-positive rate in the rapid assay appeared to be, at least in part, attributable to the increased sensitivity *in vitro* of some tumor types [2], the relative decrease in the numbers of false-positive correlations in our study might be related to the lower *in vitro* sensitivity.

Our method has several advantages over the thymidine incorporation assay or MINI-assay, including technical simplicity, shorter time course (4 days), and requirement of a smaller or the same number of cells for plating with improved or comparable evaluability rates. A greater predictive accuracy for sensitivity was provided without decreasing the predictive accuracy for resistance. Thus, this assay may be of value in chemosensitivity testing for common types of human malignant tumors, although more prospective studies will be necessary.

REFERENCES

1. Tanigawa N, Kern DH, Hisaka Y, Morton DL. Rapid assay for evaluating the chemosensitivity of human tumors in soft agar culture. *Cancer Res* 1982, **42**, 2159–2164.
2. Sondak VK, Bertelsen CA, Tanigawa N *et al.* Clinical correlations with chemosensitivities measured in a rapid thymidine incorporation assay. *Cancer Res* 1984, **44**, 1725–1728.
3. Sondak VK, Bertelsen CA, Kern DH, Morton DL. Evolution and clinical application of a rapid chemosensitivity assay. *Cancer* 1985, **55**, 1367–1371.
4. Kern DH, Drogemuller CR, Kennedy MC, Hildebrand-Zanki SU, Tanigawa N, Sondak VK. Development of a miniaturized improved nucleic acid precursor incorporation assay for chemosensitivity testing of human solid tumors. *Cancer Res* 1985, **45**, 5436–5441.
5. Akiyoshi T, Wada T, Nakamura Y, Tsuji H. A simplified method for determination of tritiated thymidine incorporation into cells from tissue in soft agar culture. *Jpn J Surg* 1986, **16**, 235–238.
6. Slocum HK, Pavelic ZP, Rustin YM *et al.* Characterization of cells obtained by mechanical and enzymatic means from human melanoma, sarcoma, and lung tumors. *Cancer Res* 1981, **41**, 1428–1434.
7. Bertelsen CA, Sondak VK, Mann BD, Korn EL, Kern DH. Chemosensitivity testing of human solid tumors. A review of 1582 assays with 238 clinical correlations. *Cancer* 1984, **53**, 1240–1245.