Jpn. J. Cancer Res. 82, 607–612, May 1991

In vitro Chemosensitivity Test of Human Gastric Carcinomas Using Collagen Gel Matrix

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We retrospectively evaluated the usefulness of an *in vitro* human tumor culture system using a specialized collagen gel matrix derived from pig skin as a chemosensitivity test for human gastric carcinomas, especially for scirrhous gastric carcinomas. Seven xenograft tumors derived from human gastric cancers were examined by using this system and the results were compared with the data obtained from a nude mouse assay. Xenograft tumors exhibited three-dimensional growth on the collagen gel matrix like that *in vivo*. The drug sensitivity as measured by this assay at 10 times therapeutic peak plasma concentrations of the drugs corresponded with that measured by the nude mouse assay for all xenograft tumors. The correlation coefficients were 0.873 for cisplatin, 0.919 for etoposide, 0.880 for mitomycin C and 0.932 for adriamycin. In the case of scirrhous gastric carcinoma, the drug sensitivity could be measured successfully in all 12 patients. This *in vitro* assay system has advantages as a chemosensitivity test because of its convenience, rapidity, and *in vivo*-like three-dimensional tumor growth. This system should contribute to the development of chemotherapy for scirrhous gastric carcinomas.

Key words: Chemosensitivity test — Collagen gel matrix — Pig skin — Human gastric cancer — Scirrhous gastric cancer

Improved prognosis for patients with malignant disease requires the development of methods for rapid and accurate prediction of clinical response to specific chemotherapeutic agents. Scirrhous gastric carcinoma is one of the most notable human gastric carcinomas, because of the rapidity of progress of the disease,[1] the difficulties of treatment,[2,3] and the high mortality.[4] Furthermore, it is difficult to find anti-cancer drugs that may prove effective on scirrhous gastric carcinomas, because published *in vitro* chemosensitivity tests[5–9] require dissociation of cells, and it is extremely difficult to dissociate scirrhous gastric carcinomas to get viable cancer cells.

Freeman and Hoffman[10] reported that tumors isolated at surgery grew with a high frequency and maintained their important *in vivo* properties *in vitro*, including the tissue architecture, in a collagen gel-supported culture system. They also reported that *in vivo*-like drug responses of human tumors could be obtained in this assay.[11]

In this study, we improved their method, retrospectively evaluated its usefulness as an *in vitro* chemosensitivity test, and used it for chemosensitivity testing of scirrhous gastric carcinomas.

MATERIALS AND METHODS

Human gastric carcinomas The tumors used in this study were 7 xenograft tumors derived from human gastric carcinomas that were established in the Department of Surgery II of this university. These tumors were maintained by serial subcutaneous transplantation of a 2-mm cubic fragment in the right subaxillary region of athymic BALB/c *nu/nu* mice (Clea Japan, Inc., Tokyo). Six- to eight-week-old female athymic nude mice weighing about 20 g were used. Mice were housed under specific pathogen-free conditions. The tumors resembled the original one histologically. Twelve specimens of scirrhous gastric carcinomas obtained from patients, who had been diagnosed and treated in this Department of Surgery II, during the period from January to October 1990, were also examined in this study.

Collagen gel matrix (CGM) assay A 1-cm cube of specialized collagen gel matrix derived from pig skin (Spongostan, Health Design Industries, Rochester, NY) was placed in each well of a 24-well tissue culture plate (Costar, Cambridge, MA) and then hydrated adequately with RPMI 1640 medium (Nissui Pharmaceutical Co. Ltd., Tokyo), supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY). The tumors obtained from nude mouse or patient were cut into thin fragments (2×2×1 mm): for equal incorporation of MTT inside and outside solid tumor fragments, the tumor tissue fragments should be less than 1 mm in thickness. They were weighed, placed on the matrix and incubated at 37°C in a humidified CO_2 incubator. At that time, the top of the matrix and the tumor fragment were not covered with the medium. Four days later, the medium was replaced with fresh medium containing the

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drugs. They were used at the following therapeutic peak plasma concentrations: cisplatin (CDDP), 2.5 μg/ml; etoposide (VP-16), 34.0 μg/ml; mitomycin C (MMC), 1.5 μg/ml; and adriamycin (ADM), 0.6 μg/ml as estimated by Scheithauer *et al.*[12] and 10-fold equivalents. The plate was incubated for 3 more days. To evaluate the viability of the tumor fragment, 100 μl of PBS solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)[13] (Sigma Chemical Co., St. Louis, MO) at the concentration of 1 mg/ml and to count the tumor cells that had invaded the matrix, 50 μl of 0.5% collagenase (Worthington Biochemical Co., Freehold, NJ) solution were added to digest the collagen gel matrix in each well. After incubation for 4 h, the plates were centrifuged at 1500 rpm for 10 min and the supernatants were discarded. The resulting MTT-formazan crystals were dissolved in 1 ml of dimethyl sulfoxide (Wako Pure Chemical Industries Ltd., Osaka) and allowed to stand overnight in a dark place at room temperature. Two hundred μl of the MTT-formazan solution was pipetted to the 96-well flat-bottomed micro plate and the absorbance at 540 nm was automatically measured by a scanning multiwell spectrophotometer (Immunoreader NJ-2000, Japan Intermed Co. Ltd., Tokyo). The drug response rates were calculated from the absorbance adjusted for weight.

Cell cycle on the collagen gel matrix Cell cycle analysis of the tumor cells on the collagen gel matrix was done with an EPICS C flow cytometer (Coulter Electronics, Inc., Hialeah, FL) on day 0, day 1, day 4, and day 7. The tissues were fixed with 70% cold ethanol for 1 h and then digested enzymatically with 0.5% pepsin solution (pH 1.5) for 2 h. After filtration through a 40-μm nylon monofilament mesh, the cells thus obtained were washed twice in phosphate-buffered saline (PBS)/0.5% Tween 20 and stained with 1 ml of PBS/0.5% Tween 20 containing 20 μg/ml propidium iodide (Sigma Chemical Co.) for 15 min.

Nude mouse (NM) assay The tumor mass was excised and cut into about 3 mm$\times$3 mm pieces. Then fragments were implanted subcutaneously. When the tumor volume reached 100–300 mm^3, the mice were randomly assigned to several experimental groups, each consisting of 6 mice, and the drugs were administered. The doses were as follows: CDDP, 8 mg/kg; VP-16, 15 mg/kg; MMC, 6 mg/kg; and ADM, 8 mg/kg. CDDP, VP-16, and MMC were injected intraperitoneally, and ADM intravenously.[14] The tumor volume (V) was calculated for an ellipsoid by using the formula $V=(a\times b^2)/2$, where a and b are the measurements (in mm) of length and width, respectively. Each tumor volume was then expressed as the relative tumor volume (RV) calculated by the formula $RV=V_n/V_0$, where V_n is the mean tumor volume at day n and V_0 is the mean initial tumor volume at the start of treatment (day 0). The effectiveness of each drug was evaluated in terms of the T/C (%) ratio (mean tumor volume of the treated tumors/that of the control$\times$100) at day 21. T/C was expressed as the average of RV of the treated mice with respect to the control. Evaluation as effective was based on an inhibition rate of 58% or more, which was calculated by using the formula $(1-T/C)\times 100$, with statistical significance as measured by the Mann-Whitney U-test ($P<0.01$, one-sided).[14]

Histological study of gastric carcinomas on the collagen gel matrix To evaluate the viability of tumor cells on the collagen gel matrix, 40 μM bromodeoxyuridine (BrdUrd) was added to the medium on day 7 and incubated for 24 h. Each tumor fragment on the collagen gel matrix was fixed with 10% neutral buffered formalin and embedded in paraffin. Sections were stained with anti-BrdUrd monoclonal antibody (Becton Dickinson, Mountain View, CA) by the avidin-biotin-peroxidase complex method using a Vectastain ABC kit (Vector Lab., Burlingame, CA). The sections were also stained with hematoxylin and eosin, and studied microscopically.

RESULTS

Effects of tumor weight on MTT-formazan production To examine the relationship of tumor weight to the production of MTT-formazan, tumors weighing from 2 to 100 mg were plated. MTT was immediately added and incubated overnight. Plates were then processed as described in "Materials and Methods," and the absorbance at 540 nm was measured. Figure 1 shows the

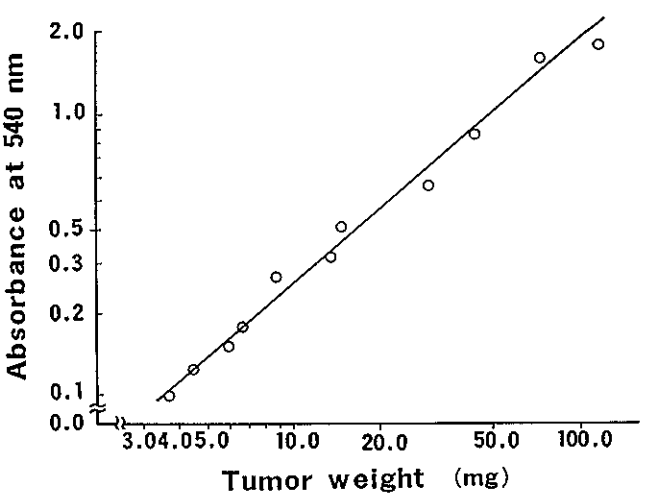

Fig. 1. Relationship between tumor weight (mg) and the absorbance at 540 nm of MTT-formazan.

results. The amount of MTT-formazan was proportional to the tumor weight.

Tumor growth on the collagen gel matrix The growth rates of the tumor cells on the collagen gel matrix were calculated from the absorbance of MTT-formazan in each xenograft tumor over a period of 8 days. At day 1 or day 2, the viability decreased. Thereafter, the tumor cells grew well (Fig. 2). Xenograft tumors showed *in vivo*-like three-dimensional growth on the collagen gel matrix. Tumor cells invading the collagen gel matrix were also observed (Fig. 3).

Drug sensitivity of xenograft tumors in the CGM assay and its relationship to the nude mouse assay Table I shows the drug sensitivity as measured by the CGM assay and the NM assay. Increasing cell kill was found with rising cytotoxic drug concentration. Drug sensitivity as measured by the CGM assay at 10 times therapeutic peak plasma concentrations of the drugs corresponded with that measured by the NM assay: The correlation coefficients were 0.873 for CDDP (MKY in Table I was statistically withdrawn from the calculation), 0.919 for VP-16, 0.880 for MMC and 0.932 for ADM, respectively. But the drug sensitivity as measured by the CGM assay at the peak plasma concentrations of the drugs did not completely correspond with that measured by the NM assay. The correlation coefficients were 0.021 for CDDP, 0.889 for VP-16, 0.146 for MMC and 0.741 for ADM.

Cell cycle on the collagen gel matrix Cell kinetics of the tumor cells on the collagen gel matrix are shown in Table

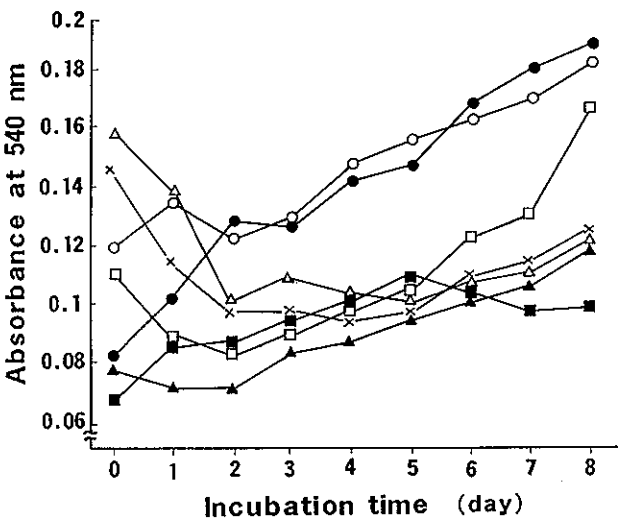

Fig. 2. Growth of the 7 xenograft tumors on the collagen gel matrix. Tumor growth was estimated by the measurement of the absorbance at 540 nm of MTT-formazan.

Fig. 3. Histological section of xenograft tumor on the collagen gel matrix. ×100 (original magnification). Hematoxylin and eosin stain.

Table I. Comparison of the Inhibition Rates (%) between the Collagen Gel Matrix (CGM) Assay at the Therapeutic Peak Plasma Concentrations (PPC) of the Drugs and 10-Fold Equivalents (10PPC), and the Nude Mouse (NM) Assay in the Xenograft Tumors

Tumor	CDDP			VP-16			MMC			ADM		
	CGM		NM	CGM		NM	CGM		NM	CGM		NM
	PPC	10PPC		PPC	10PPC		PPC	10PPC		PPC	10PPC	
MKH	38.0	75.2	68.5	27.5	37.6	53.8	45.2	71.2	79.6	22.8	53.5	88.0
MKF-1	22.6	88.7	86.0	52.1	78.5	75.5	28.3	92.6	91.3	26.2	61.5	83.1
MKF-2	51.7	82.6	81.6	39.0	75.2	93.1	40.8	83.2	98.2	29.4	75.3	90.8
MKF-3	33.0	85.0	95.6	8.6	24.8	29.8	33.6	61.8	76.3	7.8	22.6	26.5
MKK	81.8	92.3	98.6	1.8	8.3	20.7	60.4	73.5	83.1	70.2	91.8	97.6
MKY	2.0	45.7	90.8	36.5	43.1	70.7	23.8	78.0	88.2	3.8	17.2	13.1
MKS	62.5	70.9	72.0	46.5	68.5	67.1	71.6	88.3	94.6	55.0	58.6	80.2

II. There was an accumulation of tumor cells in G_2M phase in day 1. However, at day 4, the cell cycle resembled that at day 0, and it continued on further incubation.

Application of CGM assay for scirrhous gastric carcinomas Table III shows the drug sensitivity of 12 patients with scirrhous gastric carcinoma. Drug sensitivity was measured successfully in all cases. CDDP, VP-16, MMC, and ADM were evaluated as effective in 5 patients (45.5%), in 1 patient (10%), in 8 patients (66.7%), and in 5 patients (41.7%), respectively. Figure 4 shows a

Table II. Cell Cycle Analysis of the 7 Xenograft Tumors on the Collagen Gel Matrix

Cell cycle (%)	Day 0	Day 1	Day 4	Day 7
G_0G_1	59.7±5.4	50.0±4.0	59.2±5.0	60.8±3.6
S	17.3±2.4	15.1±4.2	18.9±2.0	18.4±3.3
G_2M	23.0±3.8	33.6±6.1	20.7±3.9	22.0±3.3

Table III. Inhibition Rates (%) for the 12 Patients with Scirrhous Gastric Carcinoma in the Collagen Gel Matrix Assay at 10-Fold Therapeutic Peak Plasma Concentrations of the Drugs

Patient Age Sex	CDDP (25 μg/ml)	VP-16 (340 μg/ml)	MMC (15 μg/ml)	ADM (6 μg/ml)
46 female	ND[a)]	ND	68.8	40.5
36 female	15.5	0.0	12.0	6.4
38 female	35.9	19.5	51.6	17.5
32 female	47.4	28.5	32.4	22.8
28 female	61.0	11.2	58.6	5.4
62 female	32.4	6.9	40.9	33.7
32 female	78.0	26.3	54.7	23.5
65 female	71.0	−29.5	69.4	65.5
71 female	75.8	−79.6	74.8	−114.7
69 female	31.2	−12.4	31.4	45.2
65 male	39.7	49.5	69.2	66.3
72 female	24.5	ND	37.2	77.9

a) ND, not determined.

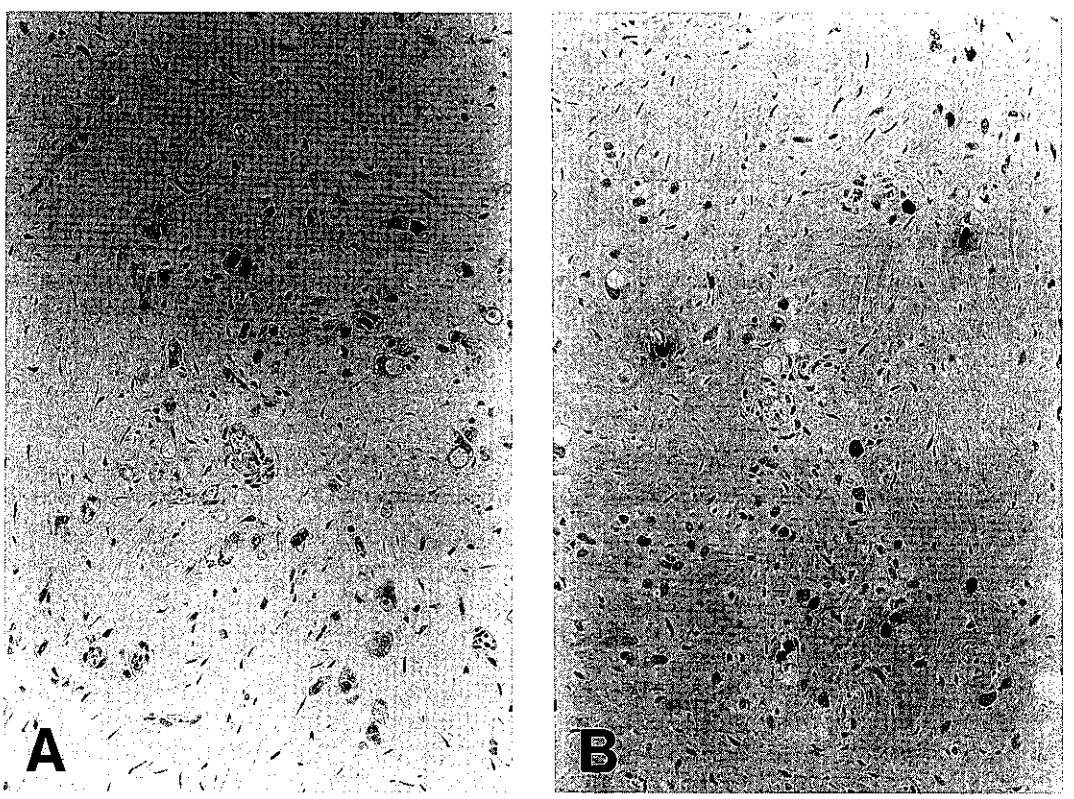

Fig. 4. Histological section of scirrhous gastric carcinoma on the collagen gel matrix. ×200 (original magnification). A; hematoxylin and eosin stain. B; immunohistochemically stained with anti-BrdUrd monoclonal antibody.

scirrhous gastric carcinoma incubated on the collagen gel matrix on day 8. There were many viable tumor cells incorporating BrdUrd.

DISCUSSION

Improved prognosis for patients with malignant disease requires the development of methods for rapid and accurate prediction of clinical responses to specific chemotherapeutic agents. A number of chemosensitivity tests,[5-9, 15, 16] either *in vitro* or *in vivo,* have been tried to predict the efficacy of anticancer agents in individual patients before administration. However, no test that accurately predicts the clinical response to cancer therapy in individual patients has been developed.

Hoffman *et al.*[10, 11] reported that important *in vivo* properties of human tumors were maintained, and *in vivo*-like drug responses could be obtained, in a collagen gel-supported system. This organ culture system has several advantages. The first is that this system meets the important criteria for *in vivo* growth. Xenograft tumors derived from human gastric carcinomas had *in vivo*-like three-dimensional growth and grew stably on the collagen gel matrix. The cell cycle of the tumor cells on the collagen gel matrix also resembled that *in vivo.*[17] Secondly, this system does not require the dissociation of cells. When cells are dissociated from solid tumors and incubated for short periods in liquid medium, they are exposed to an alien environment unlike the *in vivo* conditions. Therefore, their responses to drugs may not be relevant to those of cells *in vivo.*[18] This second advantage enabled us to examine the drug sensitivity in patients with scirrhous gastric carcinoma. The third advantage is the preservation of cellular interaction. Miller *et al.*[19] reported that the shape of the concentration-response curves for the collagen gel assay was nonexponential and that cellular interaction made tumors drug-resistant; the cellular interaction may be one of the most critical factors affecting chemosensitivity *in vivo.*[20, 21]

We improved this culture system to make it more rapid and covenient for chemosensitivity testing, and examined whether *in vivo*-like drug sensitivity could be obtained. The first improvement was the use of MTT[14] to measure the viability of tissues, instead of ^{3}H-thymidine autoradiography[11] which is hazardous and time-consuming. In comparison with monolayer cultures, increased percentages of the cells are arrested in G_0-G_1 phases of the cell cycle in the CGM assay.[17, 22] Therefore, the use of MTT is more suited for evaluating the viability of whole tumor tissues than ^{3}H-thymidine autoradiography. The second improvement was digestion of the collagen gel matrix with collagenase. This enabled us to measure the viability of tumor cells that invaded or migrated into the collagen gel matrix as observed frequently in the CGM assay. It was supposed that these cells might have chemosensitivities different from those of the original explant.[23, 24] Therefore, it is potentially clinically important to measure the viability of tissues including these cells. These improvements enabled us to predict the chemosensitivity of tumors accurately within 8 days. The drug sensitivity at 10 times therapeutic peak plasma concentrations of the drugs corresponded with that measured by the NM assay in all xenograft tumors; the correlation coefficients were more than 0.87. This suggests that the CGM assay may be more like an *in vivo* chemosensitivity test.

We used this chemosensitivity testing system for scirrhous gastric carcinomas. Many viable cancer cells that incorporated BrdUrd were observed in the tissues cultured on the collagen gel matrix on day 8. Evaluation of drug sensitivity could be made in all cases. So it is considered that the CGM assay could be clinically useful for testing of individual human gastric cancer patients and should contribute to the development of new chemotherapy for scirrhous gastric carcinomas.

ACKNOWLEDGMENTS

We thank the Research Laboratories of Sumitomo Pharmaceutics Co., Ltd. for providing Spongostan. Part of this work was supported by a Grant-in-Aid for Cancer Research (02151021) from the Ministry of Education, Science and Culture, Japan.

(Received December 27, 1990/Accepted February 18, 1991)

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