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Original articles

Comparative study of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and tritiated thymidine in a chemosensitivity test using collagen gel matrix

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Summary. An organ culture system using collagen gel matrix (CGM) was modified and established successfully as a rapid and convenient method for determination of anti-cancer drug sensitivities. The cell viability of a tumor fragment was measured by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a colored formazan product, which allowed for quantitative and simple analysis. The sensitivities of KK-47 bladder tumor from nude mice to various anti-cancer drugs tested corresponded closely to those determined in the originial CGM assay system using tritiated thymidine, which has a high clinical correlation. Our modified method can be used as a practical and highly reproducible chemosensitivity test in vitro.

Key words: Chemosensitivity test – Collagen gel matrix – 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide – Human transitional cell carcinoma

The need for a rapid and efficient assay to assess the sensitivity of human tumors to various anti-cancer drugs has led to the development of several assays in vitro, such as the tritiated thymidine ([^{3}H]TdR) incorporation assay [13], the stem cell assay [2, 6], and the tetrazolium-based colorimetric assay [1]. These assays in monolayer culture systems, however, have suffered from an inability to maintain in vivo conditions because they involve dissociation of cells from tissue, creating conditions that do not resemble the original tumor. In general, organ culture systems for solid human tumors resemble in vivo conditions more closely than do monolayer culture systems [15].

In 1986, Freeman and Hoffman developed an organ culture system using collagen gel matrix (CGM) derived from pig skin on which human tumor tissues could grow at high frequency for a long period [4]. This culture system (CGM assay) can provide a more useful method for determining in vitro proliferation capacity for normal and tumor tissues by histological autoradiography, and also for assessing the sensitivities of tumor tissues which show in vivo-like responses to various drugs [8, 16].

Despite remarkable improvements in the CGM assay several disadvantages remain, including the laborious procedures involved, lack of quantitative analysis, and limited availability since the assay employs a radioisotope to evaluate cell viability in tumor tissues. We therefore made several attempts to overcome these disadvantages and to develop a new chemosensitivity test that was rapid, simple, and practical.

Materials and methods

Tumor

Disaggregated KK-47 bladder cells, 1×10^7 suspended in saline, derived from a grade 1 human transitional cell carcinoma with a doubling time of 23.5 h [14], were injected subcutaneously into the backs of 6- to 8-week-old female BALB/c-*nu/nu* mice (Clea Japan, Tokyo, Japan). When the size of the transplanted tumor (KK-47 tumor) reached 10–15 mm in diameter, it was removed and used for this study.

Anti-cancer drugs

The anti-cancer drugs tested were doxorubicin (DOX), cisplatin (CDDP), etoposide (VP-16), methotrexate (MTX), and vinblastine (VLB), all of which were purchased commercially. Drug concentrations tested were determined by means of the therapeutic peak plasma concentrations (PPC) [12]. The PPCs of DOX, CDDP, VP-16, MTX, and VLB are 0.6, 2.49, 34.18, 2.75, and 0.78 μg/ml, respectively. Concentrations of 10 × PPC, 1 × PPC, and $^1/_{10}$ × PPC of each drug were examined.

Collagen gel matrix assay with evaluation of cell viability using MTT (the CGM-MTT assay)

A 1-cm cube of specialized CGM derived from pig skin, (Spongostan, Health Design, Rochester, N.Y., USA) was placed in each well

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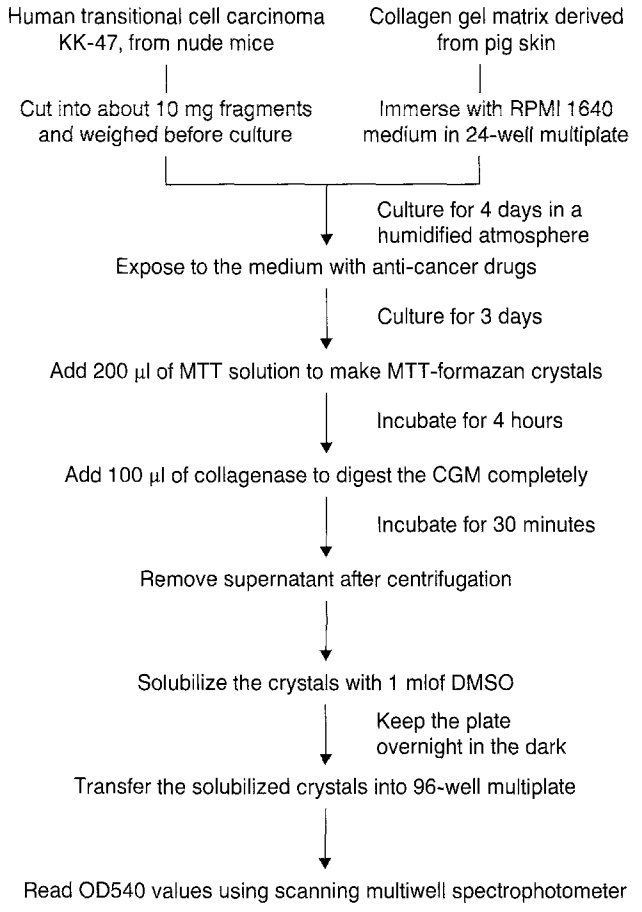

Fig. 1. Procedure for chemosensitivity test using the CGM assay with evaluation of cell viability using MTT (CGM-MTT assay)

of a 24-well multiplate (Costar, Cambridge, Mass., USA). RPMI-1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% (v/v) fetal bovine serum (Gibco, Gland Is., N.Y., USA), glutamine (Nissui) and the antibiotics penicillin G potassium and streptomycin sulfate, was added each well in sufficient quantity (approximately 2 ml) to immerse the CGM. The medium was exchanged with fresh medium until it was parallel with the CGM surface. The KK-47 tumor was cut into thin fragments. In each well a weighed tumor fragment was placed on the CGM but not covered with the medium, and cultured for various periods at 37°C in a humidified CO_2 incubator.

To evaluate the cell viability of the fragments, 200 µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, Mo., USA) solution (2 mg/ml, in phosphate-buffered saline) was added to each well (final MTT concentration 200 µg/ml), and the plate incubated for 4 h. The CGM in each well was digested with 100 µl 0.5% (w/v) collagenase (collagenase type I, specific activity 138 U/ml, Worthington Biochemical, Freehold, N.J. USA) solution to evaluate the cell viability of the tumor cells grown in the CGM. After incubation for 30 min, the plate was centrifuged at 3000 rpm for 10 min. The supernatant in each well was aspirated completely, taking care not to disturb the MTT-formazan crystals that had been produced at the bottom of the well. The plate was kept overnight in the dark at room temperature after adding 1 ml dimethylsulphoxide (DMSO, Wako Pure Chemicals, Osaka, Japan) to solubilize the crystals. Then 200 µl of the solubilized crystals in DMSO was transferred to each well of a 96-well multiplate (Costar) and the plate read at 540 nm automatically on a scanning multiwell spectrophotometer (Immunoreader NJ-2000, Japan Intermed, Tokyo, Japan). Optical density calculated at 540 nm (OD_{540}) was used to quantify cell viability in each tumor fragment.

On the basis of the results of experiments to determine an adequate tumor weight and suitable drug exposure schedule (results shown in the Results section), a drug sensitivity test using the CGM-MTT assay against KK-47 tumor was carried out as shown in Fig. 1. Immediately after removing the KK-47 tumors from mice they were cut into small fragments (approximately 10 mg in weight) and weighed before culture. After 4 days of preculture the medium in each well was exchanged for fresh medium containing the anti-cancer drug at graded concentrations. After continuous drug exposure for 3 days, OD_{540} values were measured using the procedure described above. Quadruplicate wells were used for each group to be tested. The effect of each drug was expressed as a percentage of the control, calculated by the following formula: $T/C \times 100$, where T is the mean OD_{540} value of the treated group and C that of the control group.

Collagen Gel Matrix assay with evaluation of cell proliferation using [^{3}H]TdR (the CGM-[^{3}H]TdR assay)

Drug sensitivity of the KK-47 tumor was also evaluated using [^{3}H]TdR [10], with the same drugs and drug exposure schedule as in the CGM-MTT assay. To determine the cell proliferation of the KK-47 tumor fragments, the medium in each well was replaced with fresh medium containing [^{3}H]TdR (0.2 µCi/ml, specific activity 28.4 Ci/mmol; Amersham Japan, Tokyo, Japan). The cells in the fragments were labeled for 24 h at 37°C in a humidified CO_2 incubator. After labeling the medium was removed, and the tumor fragment and CGM washed three times with phosphate-buffered saline. The tumor fragments attached to the CGM were transferred to a scintillation vial (Biovial, Beckman, Fullerton, Calif., USA) and fixed with 10% trichloroacetic acid (TCA, Sigma) at 4°C overnight. After removing the TCA, the tumor fragment and CGM were solubilized with 1 ml Nuclear-Chicago Solubilizer (NCS, Amersham Japan) at 60°C in water bath, and then 4 ml scintillator (ACS II, Amersham Japan) added to each vial. Incorporation of [^{3}H]TdR was measured in a liquid scintillation counter (model SC-31, Aloka, Tokyo, Japan), and used to quantify cell proliferation in each tumor fragment. Quadruplicate wells were used for each group to be tested. The effect of each drug was expressed as a percentage of the control, calculated by the following formula: $T'/C' \times 100$, where T' is the mean cpm value of the treated group and C' that of the control group.

Histological studies

The tumor fragment attached to the CGM after culture was fixed in 10% formalin for preparation of histology slides, which were stained routinely with hematoxylin and eosin (H & E) for light microscopy.

Results

Adequate tumor weight for the CGM assay

To determine the adequate weight of a tumor fragment for the CGM assays, the relationship between the weight of the KK-47 tumor fragments and OD_{540} values was examined on days 0 and 7. The fragments were weighed just before culture on both days. As shown in Fig. 2, there was a direct proportionality up to approximately 35 mg on day 0, (correlation coefficient, $r = 0.985$), whereas a tendency to decreasing OD_{540} values was noted on day 7 at weights

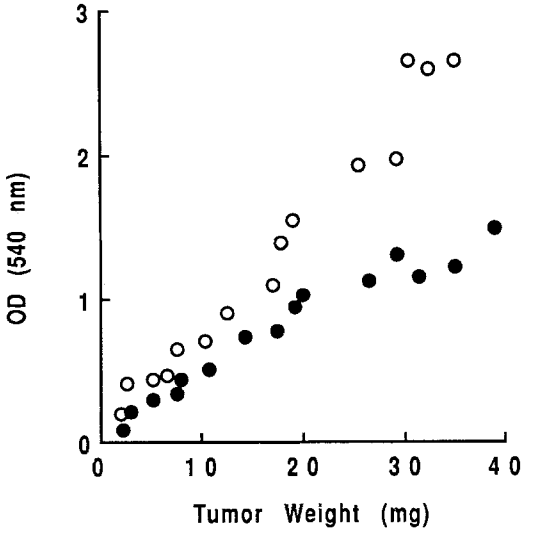

Fig. 2. Correlation between OD_{540} values and weight of KK-47 tumor fragments on day 0 (○) and day 7 (●)

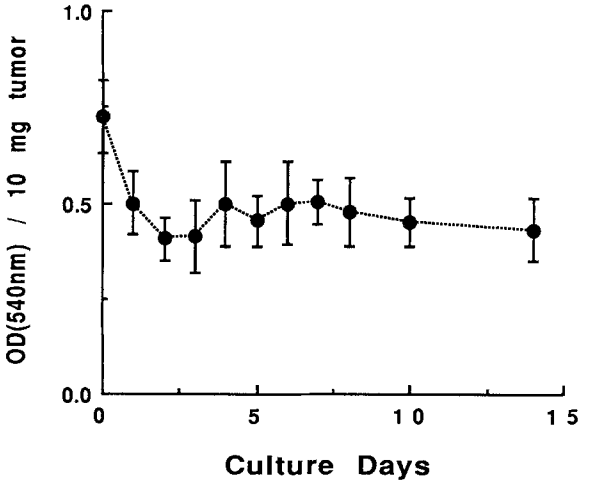

Fig. 3. Kinetics of cell viabilities in KK-47 tumor fragments after culture on CGM. Each *point* represents the mean OD_{540} value ± SD ($n = 6$)

over approximately 15 mg ($r = 0.988$). In subsequent experiments the tumor was cut into approximately 10-mg fragments, and each fragment weighed before culture. The OD_{540} value per 10 mg tumor fragment was calculated and used to quantify cell viability in each tumor fragment.

Drug exposure schedule for the CGM assay

Figure 3 shows the proliferation kinetics of the KK-47 tumor fragments. Although OD_{540} values fell rapidly until day 3, they recovered and remained stable at least during the subsequent 7-day period. In the chemosensitivity test using CGM assays, drugs were added after a 4-day preculture period and the fragments exposed to the drugs continuously for the following 3 days.

Histological findings

An H & E slide of a KK-47 tumor fragment after 7 days of culture is shown in Fig. 4a. A lack of cell viability, especially in the center, as compared with the in vivo control shown in Fig. 4b, was frequently observed even after less than 7 days of culture. In the contrast, many viable tumor cells were seen between the CGM and the tumor fragment, as shown in Fig. 5a. Moreover, many viable tumor cells were observed inside the CGM at high-power magnification (Fig. 5b) and were considered to be an invasive growth into the CGM.

Comparative sensitivities of the CGM-MTT and CGM-[^{3}H]TdR assays

The drug sensitivities of KK-47 tumor fragments were tested using the CGM-MTT and the CGM-[^{3}H]TdR assays. The results are shown in Fig. 6. Dose-dependent activities were demonstrated in all the drugs tested by both the CGM-MTT and CGM-[^{3}H]TdR assays. No significant differences between the assays were observed in the sensitivity patterns of the five drugs tested.

Discussion

The development of a highly practical in vitro chemosensitivity test to assess anti-cancer effects on human solid cancers is associated with many difficulties. We focused our attempts on the practicability of such a test in terms of simplicity and reproducibility, as well as its availability in any laboratory. We therefore applied the MTT colorimetric assay to the CGM culture system reported recently.

MTT is a good substrate in colorimetric assays and has many advantages as an in vitro chemosensitivity test [1]. The main advantage of the MTT assay is the speed with which cell viability in samples can be assessed. Another advantage of colorimetric assays is that OD values can be read without the need for removal or washing steps, which increases the speed of the assay and minimizes variability between samples. In order to introduce these advantages into the CGM assay, we studied the permeability of MTT in KK-47 tumor tissue in vitro and the solubilization of MTT-formazan with DMSO in preliminary experiments. It was observed histologically that MTT-formazan was clearly produced in the center of tissue fragments more than 10 mg in weight, as well as at the tissue periphery, after less than 4 h of contact with MTT. Complete solubilization of the MTT-formazan produced was also observed after less than 8 h of contact with DMSO. Furthermore, since direct proportionality between OD_{540} values and tumor weight was observed on day 0 (Fig. 2), we considered MTT to be applicable for evaluating cell viability without the need for dissociating tissue specimens into single cells in organ culture systems like the CGM assay.

To evaluate cell viability by MTT necessitates measuring OD values in all viable cells including those arrested in the G_0–G_1 phases of the cell cycle. In comparison with monolayer cultures, the percentage of cells in G_0–G_1 is

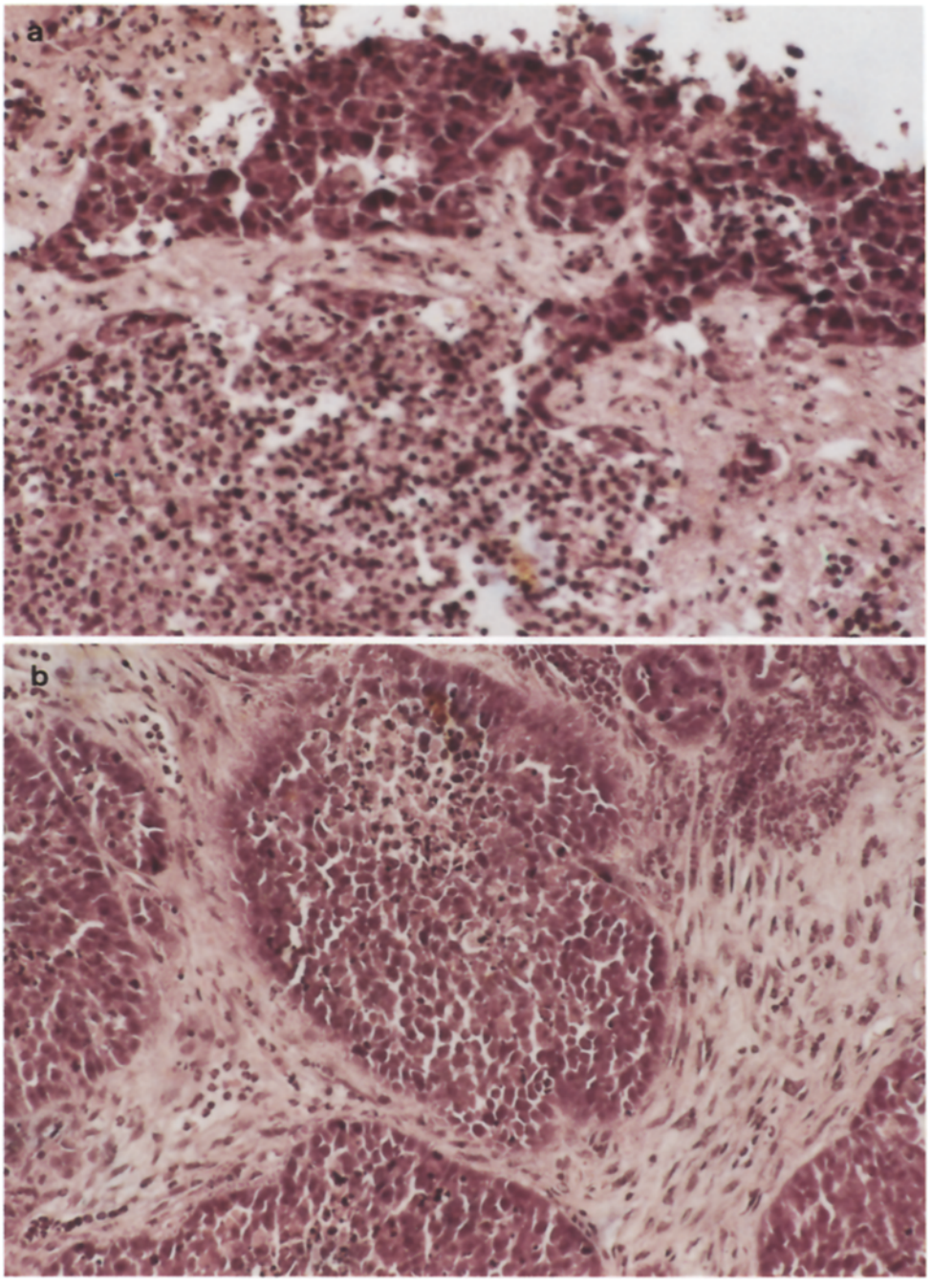

Fig. 4a, b. KK-47 tumor from nude mice stained with H & E. The tumor fragment on CGM after 7 days of culture shows necrotic change especially in the center (**a**) compared with the in vivo control (**b**). **a** ×200, **b** ×200

generally high in organ cultures [3, 5]. We therefore thought initially that using MTT in the CGM assay would be more suitable rather than using [^{3}H]TdR, which is incorporated into S-phase cells that are present in small numbers. In this regard, we recently analyzed the cell cycle of several xenograft tumors on CGM [9]. The results showed that the percentage of cells in S phase increased with continuing culture on CGM. The lack of a significant difference in sensitivity between the CGM-MTT and CGM-[^{3}H]TdR assays may be explained by this increasing percentage of S-phase cells. Since the sensitivities of the CGM-MTT and CGM-[^{3}H]TdR assays corresponded closely, the use of the former would be recommended because of its greater speed and simplicity.

Most other previously reported organ culture systems, including the watch glass technique, grid method, and some other modified methods, cannot maintain cell viability for long periods without the use of special conditions and supplementation with hormonal agents [7]. Our results, as well as those of the CGM assay using [^{3}H]TdR autoradiography, demonstrated that the cell viability of KK-47 tumor fragments was maintained for 2 weeks or more. This maintenance of cell viability was attributed mainly to cell growth between the explanted tumor and the CGM, and partly to invasive growth into the CGM. This draws attention to importance of evaluating the viability of cells inside the CGM, and taking into account to the possibility that they may demonstrate

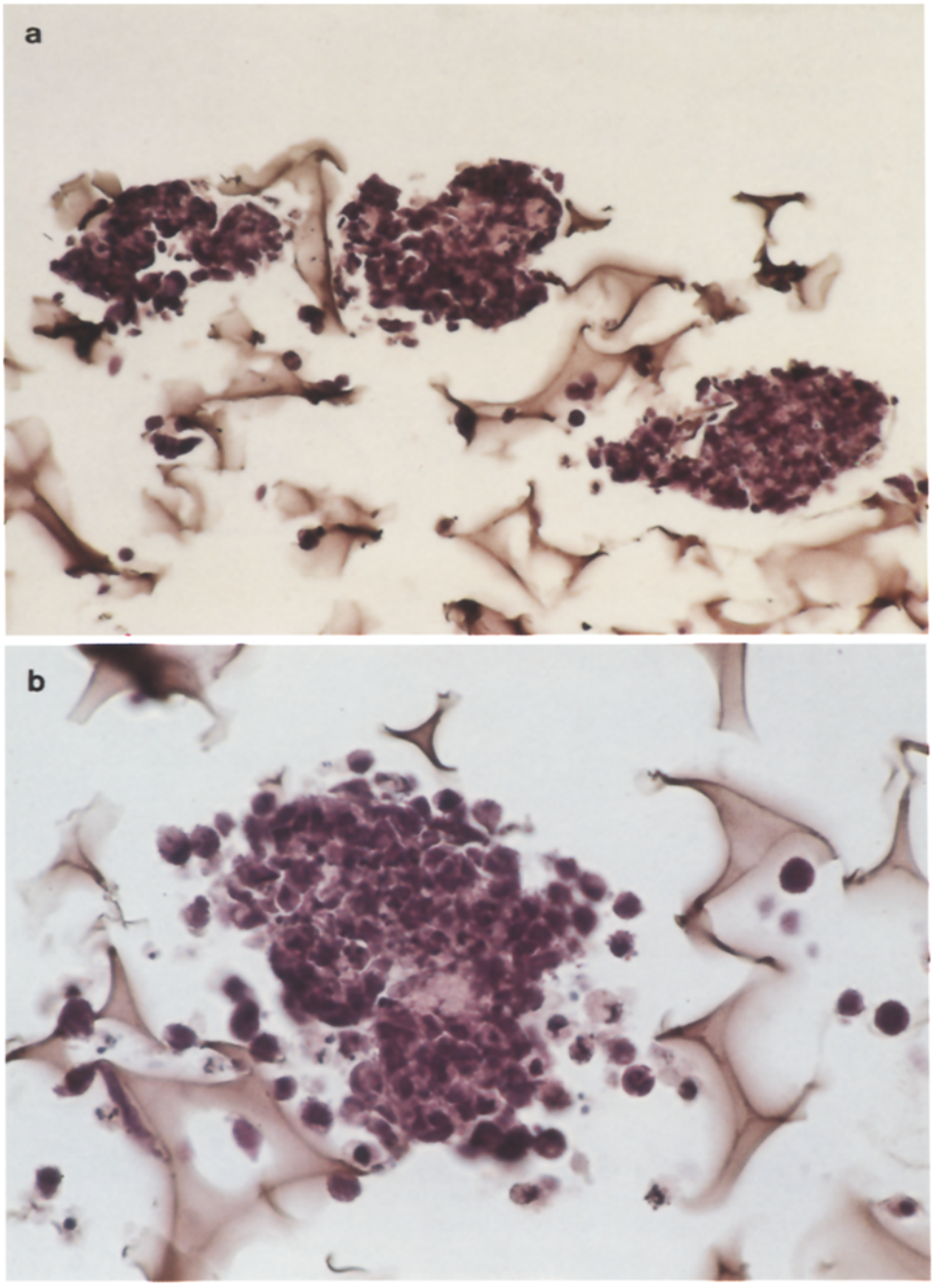

Fig. 5a, b. Many viable KK-47 tumor cells are located between the CGM and tumor fragment (**a**). Many tumor cells show invasive growth in the CGM at high-power magnification (**b**). **a** 200, **b** ×400

chemosensitivities dissimilar to those of the explanted tumor. The fact should be recognized that these cells may be important clinically in terms of invasiveness. We measured the OD_{540} values of both explanted tumors and the invasive cells in the CGM simultaneously. To measure the OD_{540} values of the cells in the CGM, they were treated with collagenase and completely digested without destroying the MTT-formazan that had been produced. It should be noted that no removal or washing steps were required during collagenase treatment.

From the point of view of practicability, it is important that chemosensitivity tests can be done and the results be provided in as short a time as possible. Our study showed that the cell viability of KK-47 tumor was maintained well, especially during the period of 4–7 days of culture. Although various schedules have been applied to chemosensitivity tests by different investigators, the timing and duration of drug exposure in our assay system were set at 4–7 days because cell viability is well maintained during this period. This schedule, together with one further day for evaluating cell viability, means the CGM-MTT assay can be completed within 8 days.

The CGM assay using [^{3}H]TdR autoradiography was reported to have a high clinical correlation [11, 16]. Our results showed that the CGM-MTT assay corresponded closely with the CGM-[^{3}H]TdR assay with respect to the chemosensitivities of the KK-47 tumor, which encourages us to apply this assay clinically. Although studies on the

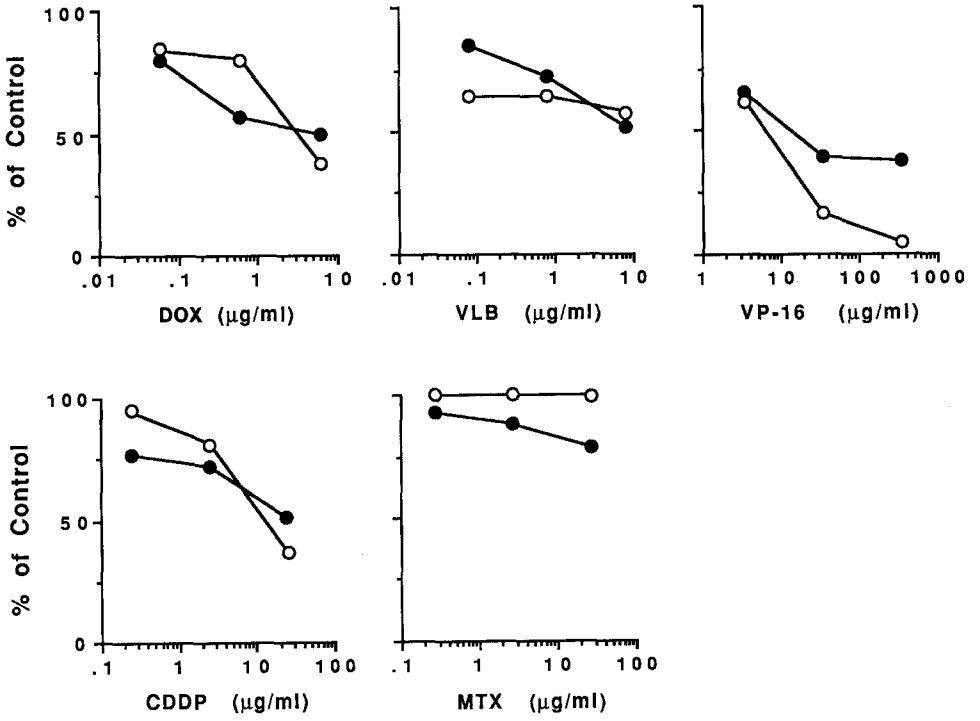

Fig. 6. Relative sensitivites of the CGM-MTT (●) and CGM-[^{3}H]TdR (○) assays. Three concentrations (10× PPC, 1× PPC, and $^1/_{10}$× PPC) of each drug were used. The effect of different concentrations of each drug was expressed as a percentage of the control, determined as described in Materials and Methods. *DOX,* Doxorubicin; *VLB,* vinblastine; *VP-16,* etoposide; *CDDP,* cisplatin; *MTX,* methotrexate

correlation between the CGM-MTT assay and clinical effects are currently continuing in our departments, the CGM-MTT assay is thought to hold considerable promise as a chemosensitivity test for individual human solid tumors because of its rapidity, convenience, and reproducibility.

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