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Examination of *in vitro* Chemosensitivity Test Using Collagen Gel Droplet Culture Method with Colorimetric Endpoint Quantification

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To develop a simpler method of performing the collagen gel droplet-embedded culture drug sensitivity test (CD-DST), we examined the introduction of colorimetric quantitative determination of images for evaluation of anticancer effect against cancer cells alone in the presence of fibroblasts, based on differences in proliferative morphology and stainability with neutral red of cells within collagen gel drops determined using a video-microscope and NIH Image software. In examinations using a human cancer cell line and a fibroblast cell line, a high degree of linearity between number of cancer cells and image-optical density was found within the range of 10^2–10^6 cells/droplet (r^2=0.933). Using NIH Image, fibroblast cells could be eliminated at a cut-off value of 128, and an immunocytochemical method demonstrated that the cells eliminated from the image were indeed fibroblasts, and those remaining were cancer cells. CD-DST was carried out with mixtures of cancer cells with fibroblasts at various ratios, and the feasibility of evaluating anticancer activity in cancer cells alone with no effect of fibroblasts at any mixing ratio was confirmed. In addition, for CD-DST of primary cell cultures of human lung cancers collected at the time of surgery, a high correlation between results obtained with the volume supplementation method, a current cell quantification method, and those with the imaging colorimetric quantification method was obtained (r=0.933). These results indicate that introduction of imaging colorimetric quantification utilizing NIH Image makes CD-DST a quick and simple method that should be highly useful for clinical chemosensitivity testing using primary cell cultures of human cancers.

Key words: CD-DST — Collagen gel droplet — Human cancers — Chemosensitivity test — Colorimetric quantification

For human malignant tumors, particularly solid cancers, the results of chemotherapy are in many cases not fully satisfactory. Therefore, it would be useful if effective anticancer drugs could be selected for each patient using screening methods. As evaluation systems for anticancer drugs, human tumor clonogenic assay (HTCA)[1–3] and succinic dehydrogenase inhibition assay (SDI)[4] have been developed, but these methods have not been practically applied to clinical cases. We therefore developed a new anticancer-drug sensitivity testing method, the collagen gel droplet-embedded culture drug sensitivity test (CD-DST), and have reported its clinical usefulness.[5–7] However, although the CD-DST method has many advantages, it requires complicated calculations to determine volume compensation values with an image-analyzing apparatus in the antitumor activity quantification step. An expensive image analysis apparatus is necessary, and this has limited the usefulness of this method in clinical laboratories. In the present study, we examined whether evaluation of antitumor efficacy is possible by using total density of neutral red incorporated by cancer cells, recorded with a video-microscope, and utilizing differences in proliferative morphology between cancer cells and fibroblasts within the collagen gel drops and differences in degree of staining with neutral red.

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MATERIALS AND METHODS

Cell lines and primary cancer cells Human lung cancer cell lines PC-13 and PC-14 were purchased from Immunobiological Laboratories (Gumma). Human lung cancer cell line A549 and human fibroblast cell line HFL-1, NB1-RGB were obtained from Riken Gene Bank (Tsukuba). Primary human lung cancer was prepared from fresh specimens extracted at the time of surgery. Each sample was treated with “Dispersion Enzyme Cocktail EZ” (including 1.0% collagenase, Nitta Gelatin Inc., Osaka). Obtained cell suspensions were inoculated into collagen-coated flasks (CG-flask, Nitta Gelatin Inc.) and cultured in pre-culture medium PCM-1 (containing insulin, epidermal growth factor, hydrocortisone, and 10% fetal bovine serum) at 37°C

in 5% CO_2 overnight. Next, collagen gel was digested with 0.05% collagenase (type I, Sigma-Aldrich Japan, Tokyo) and viable lung cancer cells were obtained.

Anticancer drugs Mitomycin C (MMC), doxorubicin (DXR) and 5-fluorouracil (5-FU) were purchased from Kyowa Hakko Kogyo, Co., Tokyo. Cisplatin (CDDP) and carboplatin (CBDCA), vindesine (VDS) and etoposide (VP-16) were obtained from Bristol-Myers Squibb Inc., Tokyo, Shionogi & Co., Ltd., Osaka and Nippon Kayaku Co., Ltd., Tokyo, respectively.

Drug sensitivity test Collagen drops were prepared as reported previously, and collagen gel droplet-embedded culture drug sensitivity tests were conducted. Briefly, "Cellmatrix" type CD, Ham's F-12 medium at 10-fold concentration and a reconstitution buffer (all from Nitta Gelatin Inc.) were mixed at a ratio of 8:1:1 to homogeneity in an ice-cold bath, and collagen solution was prepared. The cell suspension prepared above was added to this solution to make a final concentration of 1×10^5 cells/ml, affording a collagen mixture. This collagen mixture was dropped into 6-well multi-plates (Nalge-NUNC Inc., Rochester, NY) at a volume of 30 μl per collagen gel droplet using a micropipette, and subjected to gelation in a CO_2 incubator at 37°C for 1 h. In each well, 3 ml/well of DF medium (DF(10)), to which 10% fetal bovine serum had been added, was overlaid. After overnight incubation, CDDP was added at different concentrations (0.2–2.0 μg/ml). After 24 h, each well was washed with Hanks' solution twice, 4 ml/well of PCM-2 (with fetal bovine serum from removed PCM-1) medium was overlaid, and the cells were cultured for 7 days. On the 8th day of culture, 40 μl/well of neutral red solution (5 mg/ml) was added and incubation was continued for 2 h. After removal of the solution, cells were fixed with 10% formalin buffered at neutral pH. The plates were immersed in water in a tray for 10 min without agitation, and then air-dried and subjected to analysis.

Total volume quantification method As reported by Koezuka *et al.*,[8] binary images of proliferating cells in collagen gel drops were obtained with an image analyzing apparatus (VIDAS-plus, Carl Zeiss Inc., Tokyo), fibroblasts were selectively eliminated on the image, and the volume of live cancer cell colonies was calculated.

Imaging colorimetric quantification method Using a measuring apparatus consisting of a personal computer and a video-microscope, image density was measured according to the procedure shown in Fig. 1. First, an image not containing cells as a blank and an image with the light covered were obtained with a video-microscope (VH-5910, KEYENCE, Osaka), and the upper and lower limits were set. Next, the image of cells in air-dried collagen gel drops was obtained and a binary image with a cut-off at a density level of 128 (density steps: 0–255, 8 bits) was obtained. Cumulative density on the binary image was calculated, and the image-optical density (A) was obtained according to the following formula:

$$A=\log_{10}\{(\sum WC-\sum BC)/(\sum TC-\sum BC)\}$$

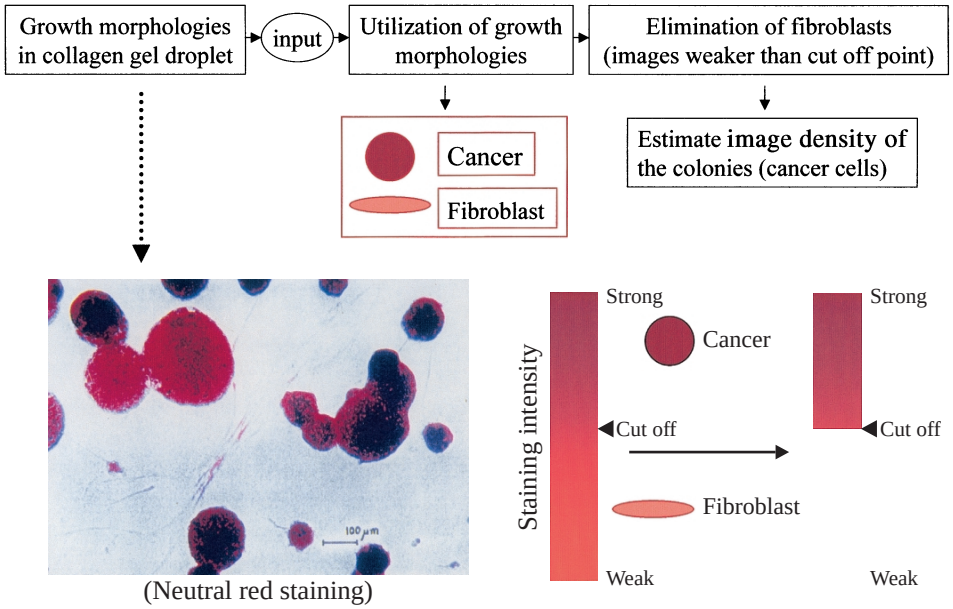

Fig. 1. Principle of measurement by the imaging colorimetric quantification method. Step 1) Acquire image of cancer cells (deeply stained) and fibroblasts (lightly stained) in collagen gel stained with neutral red. Step 2) Eliminate fibroblasts on the image by critical value procedure. Step 3) Convert image density of the remaining cancer cells on the image to optical density.

Where $\sum WC$ is the cumulative density value of the blank image, $\sum BC$ is the cumulative density value with the light covered, and $\sum TC$ is cumulative density of the test sample image.

For this processing, a personal computer (Apple Power Macintosh G-3) with grayscale image digitizer (LG-3 Image ONE, Tokyo) and "Primage" (Nitta Gelatin Inc.), a modification of the NIH Image macroprogram, were used.

Immunocytochemical staining Dispersed primary human lung cancer cells were embedded in collagen drops, cultured in PCM-2 medium for 7 days and subjected to immunocytochemical staining. After removal of culture medium, drops were covered with hydrogen peroxide, a blocking reagent, and left to stand for 5 min at room temperature for reaction. Next, after washing with purified water and a Tris-hydrochloride buffer, collagen gel drops were immersed in primary antibody solution, which was a 1:200 dilution of a 1:100 mixture of monoclonal antibodies against carcinoembryonic antigen and vimentin (Dako Japan, Kyoto) at room temperature in the native state for 30 min. Then, with biotinylated anti-mouse immunoglobin goat-antibody as a secondary antibody, antigen sites were stained with peroxidase-antiperoxidase complex and observed with a phase-contrast microscope (×100).

RESULTS

Correlation between cell number and image-optical density Using A549 cells, a calibration curve of cell density versus image-optical density obtained by the imaging colorimetric quantification method was prepared for the range of 1×10^2–1×10^6 cells/droplet (Fig. 2). A high correlation was obtained within the range of 10^3–10^6 cells/droplet (r^2=0.933).

Concentration-dependent anticancer activities of CDDP in A549 cell line at different cell densities Fig. 3 shows the concentration-dependent anticancer activity of CDDP on A549 cells at different cell densities. The 50% cell growth-inhibitory concentration (IC_{50}) was obtained for cell densities of 1–100×10^4 cells/ml (3–300×10^2 cells/droplet). In the range of 1–10×10^4 cells/ml (1.5–15×10^3 cells/droplet), the IC_{50} values were almost the same, being 3.0–3.8 μg/ml.

Elimination of fibroblasts Fig. 4 shows identification of cancer cells and elimination of fibroblasts by Primage. Each of the original images of PC-13 (large cell carcinoma) and PC-14 (adenocarcinoma) cells inputted via a video-microscope revealed good maintenance of cell shape and persistence after processing using a critical value of 128. Human fibroblasts, HFL-1 and NB1-RGB, were mostly eliminated from the image by this procedure.

Confirmation of cancer cells derived from human specimens To confirm that spherical colonies extracted at the time of quantitative analysis of images were derived from cancer cells, primary-cultured human lung cancer cells were cultured in collagen gel drops for 7 days, stained with neutral red and immunostained using anti-carcinoem-

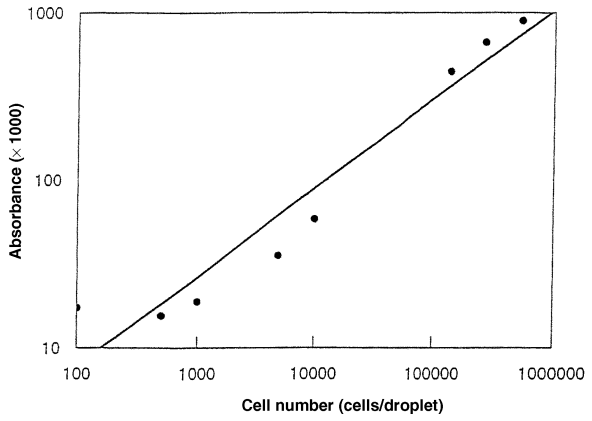

Fig. 2. Correlation between number of cells and values obtained by Primage measurement. A-549 cells were cultured at a density of 10^2–10^6 cells/droplet in DF(10) medium for 1–7 days. After neutral red staining of the cells, image-optical density was measured using Primage. Collagen gel was digested with 0.05% collagenase and treated with EDTA-trypsin, and a single cell suspension was prepared. The cells were stained with trypan blue, and viable cells were counted. r^2=0.933.

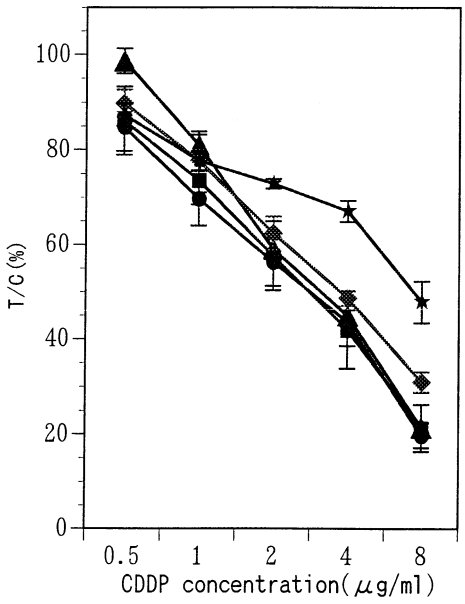

Fig. 3. CDDP dose-response curves for A-549 cells at various values of cell density. Cells were exposed to CDDP in the range of 0.1–8.0 μg/ml for 24 h. After removal of the drug, cells were cultured in PCM-2 medium for 7 days for proliferation. Experiments were carried out at cell densities of 1×10^4 (■), 5×10^4 (●), 1×10^5 (▲), 5×10^5 (◆) and 1×10^6 (★) cells/ml.

bryonic antigen (CEA) antibody and anti-vimentin antibody. Spherical colonies were densely stained with neutral red, while bipolar cells were stained faintly. Spherical colonies were densely stained with anti-CEA antibody, while bipolar cells were not stained. On the other hand, spherical colonies were not stained with anti-vimentin antibody, but bipolar cells were densely stained. Thus, spherical colonies stained densely with neutral red and identified by image analysis were confirmed to be those of cancer cells (Fig. 5).

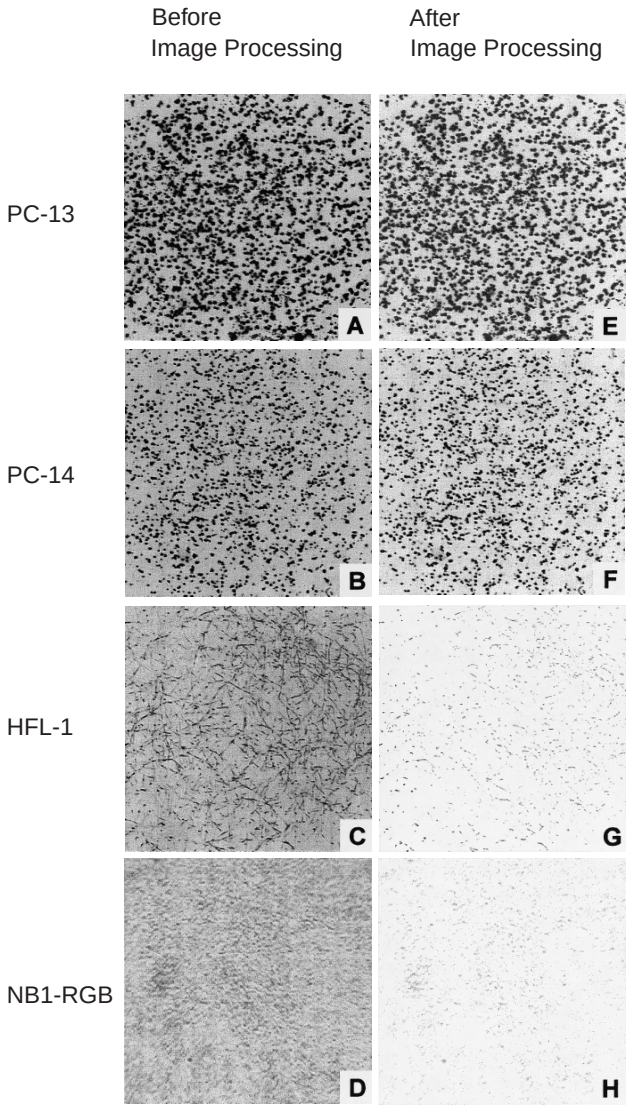

Fig. 4. Elimination of fibroblasts by Primage. Cancer cells (PC-13, PC-14) and fibroblasts (HFL-1, NB1-RGB) were embedded in collagen gel drops at a density of 1×10^5 cells/ml. The cells were cultured for 48 h in DF(10) medium, followed by culturing for 6 days in PCM-2 medium for proliferation. After neutral red staining of cells, the image was analyzed using a critical value of 128. Cancer cells exhibited no difference between the images before (A, B) and after processing (E, F), whereas the fibroblasts on images before treatment (C, D) were mostly eliminated by the processing (G, H).

Effect of mixed fibroblasts on evaluation of antitumor activity For examination of the effect of fibroblasts on quantitative evaluation of antitumor activity, mixtures of cancer cells (A-549) with fibroblasts (HFL-1) at ratios of 2:1 and 1:2 were prepared and assayed. As a control, A-549 alone was assayed, and the results of evaluation of antitumor activity were compared. In all cases, the cell density was set at 3×10^5 cells/ml. As shown in Fig. 6, as determined by image colormetric quantification, results of evaluation of antitumor activity at each mixing ratio were similar to those for A-549 alone, and similar dose-response curves were obtained. Moreover, under conditions of low sensitivity (CDDP, 0.2 μg/ml), moderate sensitivity (CDDP, 2.0 μg/ml) and high sensitivity (CDDP, 10.0 μg/ml), no effect of fibroblasts on quantitative evaluation of antitumor activity was found, and antitumor activity against cancer cells alone was evaluated.

Comparison of the volume supplementation method and imaging colorimetric quantification method for antitumor activity using primary human lung cancer cells CD-DST was performed on MMC, CDDP, VDS, VP-16, 5-FU, DXR and CBDCA using 13 fresh samples extracted at surgery. From the observed value in anticancer-drug treatment groups (T) and the observed value in the non-treatment group (C), the values of T/C (%) for the volume supplementation method and imaging colorimetric quantification method were obtained. A high correlation (r=0.933) was found between the results of these two methods for all of the anticancer drugs tested (Fig. 7).

DISCUSSION

Many chemosensitivity tests have recently been developed in attempts to ensure effective chemotherapy for each patient. *In vivo* methods, represented by the nude-mouse method[9–11] reported by Rygaard and Povlsen[12] and the subrenal capsule assay (SRC) method[13, 14] presented by Bogden *et al.*[15] have not been introduced into clinical use because of their low success rates, high cost and long time requirement. To overcome these problems, human tumor clonogenic assay (HTCA),[1–3] thymidine incorporation assay (TIA)[16–18] and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay[19, 20] have been developed as quick, inexpensive and simple *in vitro* methods. However, these methods have not been introduced into clinical use because the colony formation rate and test success rate were low with HTCA,[21] and the success rate in primary cell cultures was low with the SDI method and MTT assay. These methods are affected by contaminating fibroblasts at the time of quantification, and

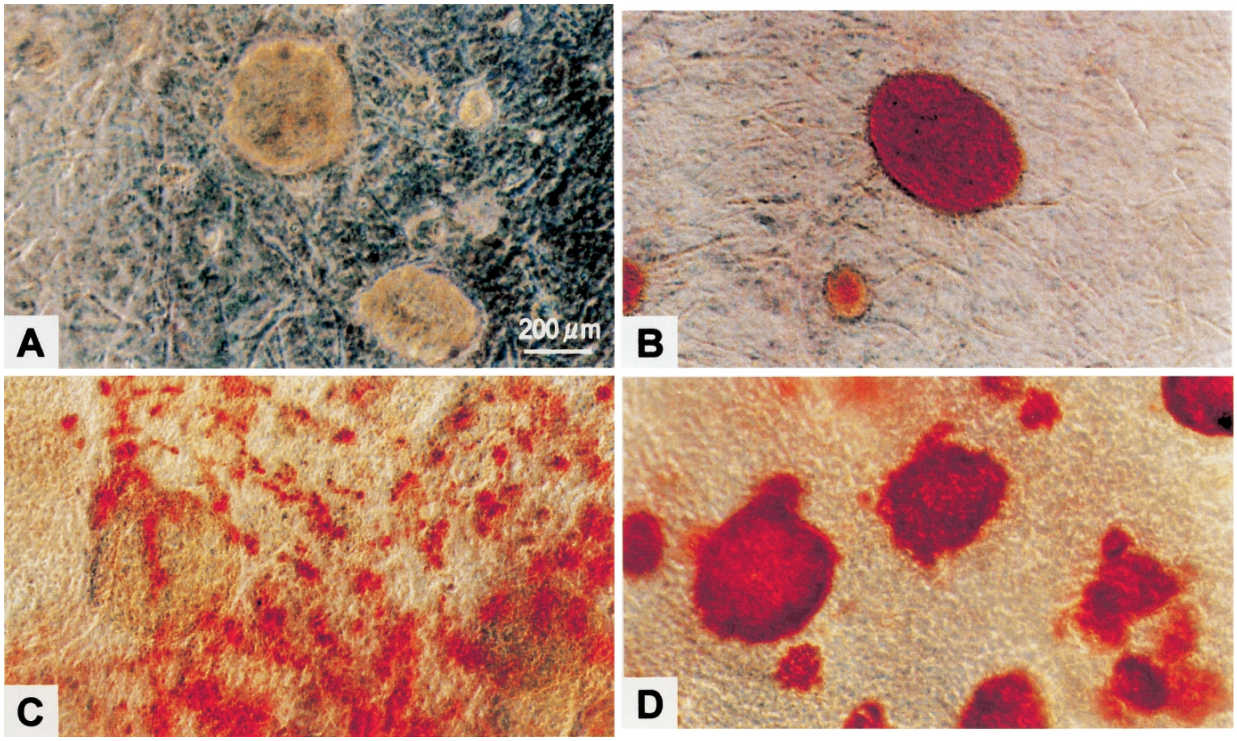

Fig. 5. Morphology of human primary lung cancer cells and fibroblasts in collagen gel droplets. A) Fixed with 10% buffered formalin. There were two types of morphology, spherical and bipolar. B) Stained with neutral red. The cancer cells were stained deeply while the fibroblasts appeared lighter. C) Stained with anti-vimentin antibody. Only the bipolar cells were stained; spherical cells were not stained. D) Stained with anti-CEA antibody. The spherical cells were stained deeply, but the bipolar cells not stained. Bar=200 μm.

are not growth assays. To solve these problems, we developed CD-DST[5] and have reported its clinical usefulness.[6, 7] For CD-DST, we used three-dimensional cultures embedded in collagen gel, using type I collagen which forms the extracellular matrix (ECM). Advantages of this culture method include its high success rate in primary cell culture, similar behavior of cells to that *in vivo*, and possible reproduction of *in vivo* phenomena.[22–25] Moreover, when this method was used for anticancer-drug sensitivity testing, antitumor activity was evaluated at drug concentrations similar to those obtained *in vivo*, and sensitivity close to that required for clinical efficacy and a high success rate of prediction of clinical efficacy have been obtained.[6] In particular, in the case of the area under the drug concentration-time curve (AUC)-dependent anticancer drugs, a high correlation of *in vivo-in vitro* behavior was obtained.[26] A histoculture method was developed by modifying a tissue culture method to make it three-dimensional with similar use of ECM, and employed for long-term culture of primary tumor cells.[27–29] Using this culture method, Hoffman *et al.*[30] and Furukawa *et al.* established the histoculture drug response assay (HDRA), and have reported its clinical utility.[31–33] However, in their method, the concentration of the applied drug was tens or hundreds

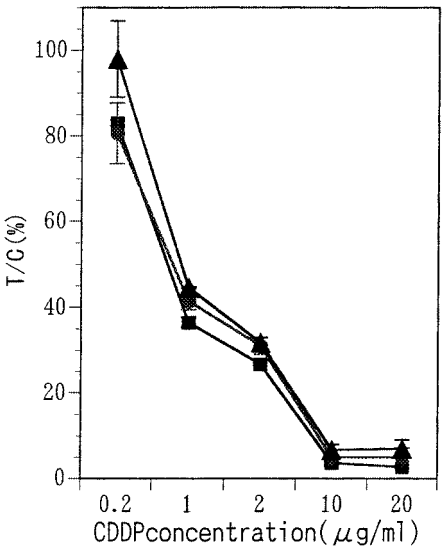

Fig. 6. Effect of contaminating fibroblasts on anticancer efficacy. Total cell density: 3×10^5 cells/ml, cancer only (◆), cancer: fibroblast 2:1 (■), 1:2 (▲). The three dose-response curves coincided well, and antitumor efficacy could be evaluated for cancer cells alone without interference from fibroblasts.

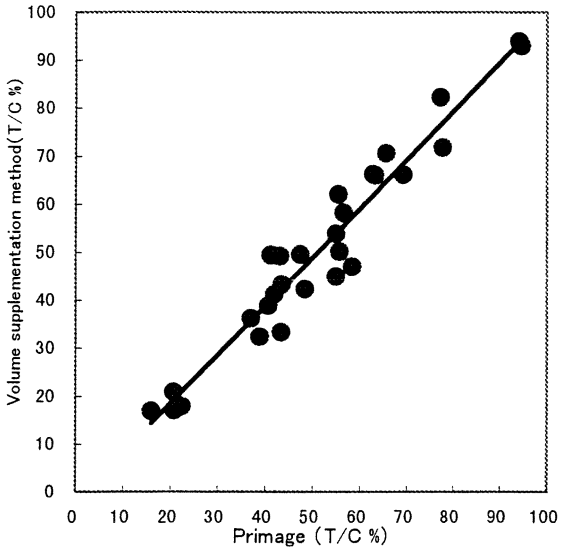

Fig. 7. Correlation between results obtained with Primage and the volume supplementation method for antitumor efficacy (T/C%). CD-DST was performed on clinical specimens of primary lung cancer collected from 13 patients. Antitumor efficacy (T/C%) obtained by the volume supplementation method and that by the imaging colorimetric quantification method were plotted for each specimen, and the correlation between the results obtained with these two quantification methods was determined. High degrees of coincidence were obtained in high, middle and low sensitivity ranges. The correlation coefficient was high, at 0.933.

of times higher when converted to AUC, whereas in CD-DST, the concentration of the applied drug is close to the *in vivo* level, which appears to be more reasonable. In addition, since MTT succinic dehydrogenase activity is used as the quantification endpoint of HDRA, succinic dehydrogenase activity in fibroblasts and other cells in tumor tissues cannot be ignored. Due to the use of the tissue culture method, its application to biopsy samples, cancerous pleural effusion or ascites is difficult, and so HDRA appears useful mainly as a sensitivity test for supplemental chemotherapy using specimens obtained during operation. CD-DST uses the collagen gel droplet-embedded culture method, and multiple tests with small numbers of cells can be performed with it. In the present study, a good correlation between observed values and number of cells was obtained over the range of $1.5–15\times10^3$ cells/droplet, and a detection rate equivalent to those obtained with existing methods was confirmed. Results of immunocytochemical examination suggested that differentiation of cancer cells from fibroblasts based on proliferative morphology may be possible. Since contaminating vascular endothelial cells cannot grow due to the use of serum-free PCM-2 medium which does not contain fibroblast growth factor (FGF)-2, and will be apoptotic,[34] antitumor activity towards cancer cells alone can be evaluated accurately. Borenfreund and Puerner[35, 36] reported that neutral red (NR) was accumulated by viable cells in lysosomes through the normal plasma membrane, while injured cells could not take up NR; hence, there was a correlation between total uptake of NR and number of viable cells. On the other hand, cancer cells, under the conditions of collagen gel droplet-embedded culture, grew in collagen gels with a three-dimensional spherical morphology, while fibroblasts, since they were healthy, exhibited bipolar extension. For these reasons, the cancer cell colonies were densely stained with NR due to piling up, while the NR-containing fibroblasts appeared lighter in color, with scattered lysosomes.

Even when cancer cells were mixed with fibroblasts, antitumor activity against cancer cells alone could be evaluated (Fig. 7), and evaluation at high or low levels of sensitivity appeared to be possible. When primary cell cultures of human cancer were used, the results of evaluation of antitumor activity showed a high correlation with those of the volume supplementation method,[6] for which a high success rate of prediction of clinical efficacy has been reported. It costs about US$100 000 to set up the conventional system (VIDAS plus etc.) for evaluation of total volume. However, it is possible to set up our new system for colorimetric endpoint quantification for about US$10 000 along with a personal computer. Regarding speed of evaluation, the former system requires about 20 min to perform 18 tests with 6 drugs, while the latter requires only 1 min.

Therefore, our results strongly suggest that this new quantification method will be excellent for prediction of clinical efficacy. It permits the evaluation of antitumor effect against cancer cells alone with high sensitivity by differentiation of cancer cells from fibroblasts using a combination of neutral red staining, NIH Image analysis software and video-microscopy, and is inexpensive and quick (about 3 s/droplet). This improvement should enable almost every laboratory to use CD-DST.

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