Improvement of in Vitro Chemosensitivity Assay for Human Solid Tumors by Application of a Preculture Using Collagen Matrix

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ABSTRACT

The use of \(^{3}H\)thymidine incorporation assay (TIA) to evaluate the drug response of tumor cells has been recognized as a useful chemosensitivity assay for fresh human tumor specimens. However, its low evaluability has been a disadvantage for clinical application. To overcome this drawback, we have applied a preculture stage prior to the TIA. This preculture requires plating the tumor cell suspension onto a collagen matrix for 24 h.

In 29 fresh human tumor specimens, a significant increase in both cell viability (\(P < 0.05\)) and \(^{3}H\)thymidine incorporation (\(P < 0.001\)) of the cultured cells was observed with preculturing; the composition of cancer cells (epithelial membrane antigen positive) and stromal cells (vimentin positive) did not change. In comparisons between 66 specimens that were precultured and 705 specimens that were not, the evaluability rate increased significantly from 48.5% (342/705) to 75.8% (50/66; \(P < 0.0001\)) after preculturing. No significant change in in vitro chemosensitivities was observed. When the clinical responses for cancer chemotherapy were retrospectively compared with those in vitro, sensitivities were selectively cultured using a preculture system composed of the extracellular matrix such as collagen (10, 11). In contrast, a soft agar culture system has been shown to be suitable for culturing a variety of solid tumors while suppressing in vitro growth of fibroblasts (12). However, one drawback of the soft agar culture system is its low evaluability, which can make it difficult for application in clinical chemotherapy (13–20).

Application of the TIA has been developed from the double agar system (a clonogenic assay) to attain higher rates of evaluability and provide a rapid assessment of chemosensitivities (21–25). However, our study on gastrointestinal cancers resulted in insufficient rates of evaluability, which inhibited a broader application of this assay for clinical chemotherapy (26). In vitro culture systems using extracellular matrix are not only useful for culturing tumor tissue in pseudo in vivo conditions but also favorable for attaching viable cells to the matrix (27). Accordingly, in the present study, we report how preculture influences the evaluability rates and drug sensitivity profiles in TIA. We also investigate the applicability of combining preculturing with TIA for determining clinical chemotherapy.

MATERIALS AND METHODS

Specimens. Sixty-six fresh tumor specimens removed from patients from October 1994 to March 1996 at The Second Department of Surgery of Fukui Medical School were used in this study. They included carcinomas of 17 gastric, 9 colorectal, 16 lung, 4 breast, 9 ovarian, 1 pancreatic, and 4 yolk sac tumors as well as 1 malignant melanoma and 5 primary unknown tumors.

The difference in cell viability, composition of cancer and stromal cells, and \(^{3}H\)thymidine uptake without and with preculture were examined in 29 of the 66 specimens, consisting of 13 gastric, 3 colorectal, 6 lung, 4 breast, 2 ovarian, and 1 pancreatic carcinomas.

TIAs combined with a preculture were performed on a total of 66 specimens, and the rates of evaluability and chemosensitivity against anticancer agents were compared with 705 specimens that were not precultured. They included 206 gastric, 115...
colorectal, 64 lung, 10 breast, and 33 ovarian carcinomas and 277 other tumors. In addition, the correlation between results of the in vitro assay and subsequent clinical responses was investigated in 16 patients who had measurable lesions, including 5 gastric, 1 colorectal, 3 lung, 3 ovarian, and 1 breast carcinomas, 2 yolk sac tumors, and 1 malignant melanoma.

**Preparation of Fresh Tumor Cells.** Tumor tissues obtained from the resected specimens were washed with saline and mechanically minced. The minced tissues were disaggregated by stirring in HBSS supplemented with 0.03% DNase I (Sigma Chemical Co., St. Louis, MO) and 0.14% collagenase type I (Sigma Chemical Co.) at 37°C for 60 to 90 min. Cells were then collected by filtration through two sheets of sterilized gauze and centrifugation.

**Preculture.** Cells were placed in 0.24% collagen gel-coated 25-cm² flasks (CG flask; Nitta Gelatin, Inc., Osaka, Japan) with DMEM (Life Technologies, Inc., Grand Island, NY) and Ham’s F12 (Life Technologies, Inc.) mixed media containing 10% FCS, 5 mg/ml epidermal growth factor (Sigma), 10 mg/ml insulin (Sigma), 20 mg/ml hydrocortisone (Sigma), 100 units/ml penicillin (Cosmo Bio, Tokyo, Japan), 100 units/ml streptomycin (Cosmo Bio), and 1.25 mg/ml amphotericin B (Fungizone; Life Technologies, Inc.). After 24 h of culture in a humidified 5% CO₂ atmosphere at 37°C, the supernatant was removed and the cells attached on the collagen gel were harvested by enzymatic treatment with 0.1% collagenase I for 60 min.

**Assessment of Cell Viability and Immunocytochemical Staining.** Cell viability of each tumor cell suspension was assessed before and after preculturing by trypsin blue exclusion testing. Cells (1 X 10⁶) were spun onto a slide and fixed with Cytokeep (Kanae Inc., Osaka, Japan). The slides were incubated overnight at 4°C with anti-vimentin (Vim 3B4; DAKO Japan) or anti-EMA (E29; DAKO Japan) antibodies at a 1:400 dilution or 1:200 dilution, respectively. Then the slides were stained using the labeled streptavidin-biotin method (DAKO LSAB kit; DAKO Japan) and counterstained with hematoxylin. Anti-mouse immunoglobulin (MsIgG2a; Coulter Immunology, Hialeah, FL) was used as a negative control in this study. Stained cells were counted on five fields at a magnification of ×200 using a microscope. The distribution of epithelial tumor cells (EMA positive) and stromal cells (vimentin positive) was evaluated by calculating the percentage of positively stained cells among the total cells.

**Application of a Preculture to TIA.** The procedure for TIA has been described previously (20, 28). Briefly, tumor tissues were disaggregated mechanically and enzymatically. After the dissociated cells were harvested, cell yield and viability were counted by trypsin blue exclusion. The cell suspension of 0.5 ml (1.5 X 10⁶ cells/well) supplemented with 0.3% agarose, which consisted of Chee’s essential medium containing 15% FCS, was poured onto each well, and the plates were returned to the incubator for an additional 24 h. Incorporation of [³H]thymidine by tumor cells was measured as described (21).

An assay was valid if the average count of the untreated controls was ≥1000 cpm and the HgCl₂ (100 μg/ml) treated control showed at least 80% inhibition compared with the untreated control. As a definition of sensitivity to anticancer drugs, ≥50% inhibition of thymidine incorporation at 0.1 X PPC or ≥80% inhibition at 1.0 X PPC was used because it had been confirmed as an appropriate cutoff for predicting clinical responses in our previous study (14, 23, 26).

**Clinical Correlations of in Vitro Results.** Clinical responses for cancer chemotherapy were retrospectively compared with the in vitro sensitivities to the corresponding drugs in 16 patients who had measurable lesions. Clinical response was defined according to the following criteria: complete response was defined as complete disappearance of measurable lesions, partial response was defined as reduction in measurable lesion volume of 50% or more, and minor response was defined as reduction in measurable lesion volume of 25–50%. These clinical responses were required to last for at least 1 month. In contrast, progressive disease was defined as progressive or recurrent disease, and no change was defined as reduction in measurable lesion volume of 25% or less.

Patients achieving clinical responses when treated with two or more drugs that were active in vitro were considered to have responded only to the most active in vitro agents. Conversely, patients with no responses who received multiple agents that had no in vitro activity were considered to have true negative correlations to each agent administered (14, 21, 26).

**Statistical Analysis.** Significant differences were determined using the Wilcoxon signed rank test and the ch₂ test. A P < 0.05 was considered to be statistically significant.

**RESULTS**

**Differences in Cell Viability, Cellular Composition, and [³H]Thymidine Uptake by Cultured Cells before and after the Preculture.** Figs. 1 and 2 show the difference in cell viability and [³H]thymidine uptake by cultured cells before and after the preculture. Both cell viability and [³H]thymidine uptake by cultured cells increased significantly by application of the preculture. Both cell viability and [³H]thymidine uptake by cultured cells increased significantly by application of the preculture (P < 0.05 and P < 0.001, respectively; Figs. 1 and 2).

Table 1 shows the differences in distribution of EMA-positive cells and vimentin-positive cells both before and after the preculture period. The distribution of cells did not change by application of the preculture.

**Assay Evaluability.** The mean [³H]thymidine uptake by cultured cells in 66 samples that had been precultured was compared to the one in 705 samples that had not undergone preculturing. In the latter, 3,360 ± 7,817 cpm, including 2,605 ± 6,356 cpm for 206 gastric carcinomas; 3,040 ± 5,809 cpm for 115 colorectal carcinomas; 2,472 ± 3,398 cpm for 64 lung carcinomas; and 10,166 ± 15,049 cpm for 33 ovarian carcinomas were obtained. In the former, 6,294 ± 12,665 cpm, including 3,040 ± 5,809 cpm for 16 lung carcinomas; and 10,166 ± 15,049 cpm for 33 ovarian carcinomas were obtained. Therefore, [³H]Thymi-
dine uptake was increased by application of the preculture in most types of tumors, except ovarian carcinomas.

The elevation of thymidine uptake resulted in increased assay evaluability. The current study demonstrated that the rates of evaluability in TIA increased significantly from 48.5% (342/705) to 75.8% (50/66) by application of the preculture (P < 0.0001; Table 2). This trend was confirmed in 29 paired tumor specimens, i.e., 44.8% (13/29) without preculturing and 72.4% (21/29) with preculturing, respectively.

**Table 2** Increase of evaluability rates by application of a preculture

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Without preculture (%)</th>
<th>With preculture (%)</th>
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<tbody>
<tr>
<td>Gastric cancer</td>
<td>34/88 (40.0)</td>
<td>11/17 (64.7)</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>57/115 (49.6)</td>
<td>8/9 (88.9)</td>
</tr>
<tr>
<td>Lung cancer</td>
<td>36/64 (56.3)</td>
<td>13/16 (81.3)</td>
</tr>
<tr>
<td>Ovarian cancer</td>
<td>25/33 (75.8)</td>
<td>7/9 (77.8)</td>
</tr>
<tr>
<td>Other tumors</td>
<td>136/287 (47.4)</td>
<td>11/15 (73.3)</td>
</tr>
<tr>
<td>Total</td>
<td>342/705 (48.5)</td>
<td>50/66 (75.8)</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SD. α, P < 0.001; β, P < 0.0001 using the χ² test.

**Discussion**

Many investigators have addressed several problems associated with in vitro assays for human tumors. MTT (1) and ATP assays (29), using monolayer cultures, are simple and rapid methods and have been broadly used in the field of hematolog-

**Table 1** Influence of preculture on cellular composition between vimentin-positive cells and EMA-positive cells

<table>
<thead>
<tr>
<th></th>
<th>Before preculture</th>
<th>After preculture</th>
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<tbody>
<tr>
<td>EMA-positive cells</td>
<td>(n = 29)</td>
<td></td>
</tr>
<tr>
<td>40.1 ± 21.2</td>
<td>39.6 ± 16.7</td>
<td></td>
</tr>
<tr>
<td>Vimentin-positive cells (n = 29)</td>
<td>64.3 ± 20.6</td>
<td>63.5 ± 18.6</td>
</tr>
</tbody>
</table>

α Each value represents the mean ± SD of the rates of vimentin- or EMA-positive cells against total cells in the cell suspension before and after preculture. The rates of vimentin- or EMA-positive cells in cell suspension were compared between groups before and after preculture. There was no significant difference assessed by the Wilcoxon signed rank test.

**In Vitro Sensitivity.** The frequencies of in vitro sensitivity to various drugs were compared between TIAIs, with and without the preculture. The percentage of inhibition of [3H]thymidine uptake induced by various drugs at 1.0 × PPC are shown: 34.8 ± 27.2% (27 testings) and 18.3 ± 21.8% (126 testings) for mitomycin C, 29.0 ± 24.6% (27 testings) and 28.4 ± 26.6% (18 testings) for cisplatin, 25.5 ± 32.0% (16 testings) and 14.4 ± 23.6% (130 testings) for 5-fluorouracil, 28.4 ± 26.6% (18 testings) and 23.2 ± 25.4% (116 testings) for doxorubicin, and 43.2 ± 27.7% (12 testings) and 25.7 ± 22.8% (24 testings) for vincristine. The values for TIA with preculture preceded the values without preculture. However, when the cutoff line was set at 50% inhibition at 0.1 × PPC and 80% inhibition at 1.0 × PPC, in vitro responses against various drugs did not significantly differ between the two assays (Table 3).

**In Vitro Vivo Correlations.** The correlation between in vitro drug responses and in vivo responses to chemotherapy with the corresponding drugs was examined in 16 patients who had evaluable lesions. There were 19 in vivo-negative responses in 20 tumors resistant in vitro, whereas there were 5 in vivo responses in 6 tumors that had been defined as sensitive in vitro. Thus, total accuracy for predicting in vivo responses was 92.3% (24/26) when the preculture was applied before starting the TIA (Table 4).
Improvement of Chemosensitivity Assay by a Preculture

Table 3  In vitro responses to various drugs in the TIA with and without preculture

<table>
<thead>
<tr>
<th>Drug</th>
<th>Without preculture (%)</th>
<th>With preculture (%)</th>
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<tr>
<td>Cisplatin</td>
<td>81/474 (~17.1)</td>
<td>7/56 (12.5)</td>
</tr>
<tr>
<td>Doxorubicin</td>
<td>35/436 (8.0)</td>
<td>5/37 (13.5)</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>45/498 (9.0)</td>
<td>4/26 (15.4)</td>
</tr>
<tr>
<td>5-Fluorouracil</td>
<td>148/498 (9.6)</td>
<td>3/36 (8.3)</td>
</tr>
<tr>
<td>Vincristine</td>
<td>62/359 (17.3)</td>
<td>5/16 (31.3)</td>
</tr>
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* In vitro response/test. In vitro sensitivity was defined as ≥80% inhibition of \[^{3}H\]thymidine uptake at 1.0 × PPC. There was no significant difference between the two groups for each drug tested.

Table 4  In vivo/in vitro correlation of TIA with preculture

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<tbody>
<tr>
<td>26</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>19</td>
</tr>
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</table>

* Prediction accuracy for sensitivity, 83.3% (5/5 + 1). Prediction accuracy for resistance, 95.0% (19/19 + 1).

* Sens/Sens, patients who were sensitive both in vitro and in vivo; Sens/Res, patients who were sensitive in vitro but resistant in vivo; Res/Sens, patients who were resistant in vitro but sensitive in vivo; Res/Res, patients who were resistant both in vitro and in vivo.

TIA was developed from the double agar culture system (21–25, 35, 36). Therefore, the main advantage of this assay is that the agarose allows tumor cells to proliferate and inhibits the growth of fibroblasts. One significant problem associated with clonogenic assays is that it is difficult to discriminate between true colony growth from a single cell and an aggregate of cells plated at the outset (18, 37, 38). However, since it is not necessary in TIA to dissociate tumor specimens into single cells, cells for testing in TIA can be plated as small clumps, maintaining cell to cell interaction, and grow three-dimensionally in double agar. In the experiment of Graham et al. (39) using the EMT-6 tumor cells, it was demonstrated that the drug resistance phenotype manifested in vitro was restored by culturing as three-dimensional multicellular aggregates. In contrast, disaggregation and culturing as a monolayer resulted in the loss of the drug resistance phenotype. This suggests that drug resistance acquired in vitro can be restored in TIA. Although the evaluability rates of TIA have increased in comparison to those of clonogenic assays (22), our previous study on gastrointestinal carcinomas indicated that the evaluability was still insufficient to apply to clinical chemotherapy (43% for gastric carcinomas and 56% colorectal carcinomas; Ref. 26). Accordingly, the low evaluability is still one of the major drawbacks for clinical application of TIA.

To increase the rate of evaluability, higher numbers of viable cells should be used for in vitro testing. However, in cell suspensions derived from fresh tumor tissues, viable cells have always coexisted with low viable and dead cells. It has been noticed that viable cells usually have a high affinity to the collagen matrix (27). We demonstrated in this study that a population of higher viable cells were obtained by harvesting cells that were attached onto collagen gel-coated flasks. \[^{3}H\]thymidine incorporation of these precultured cells was significantly increased.

Cellular composition of the tumor cell suspension was determined by immunocytochemistry using anti-vimentin and anti-EMA antibodies before or after the preculture. Vimentin is broadly used as a specific antigen for cells of mesenchymal origin (fibroblasts, smooth muscle cells, endothelial cells, and lymphoid cells), whereas EMA is specific for cells of epithelial origin. Therefore, their antibodies have been described as useful tools in distinguishing between stromal and cancer cells. Our results showed that the cellular composition of stromal and epithelial cells was approximately 60% and 40%, respectively, before the preculture, whereas the composition had not significantly changed after the preculture. The fact that stromal cells constituted 63.5% after the preculture probably indicates the need of an assay system in which tumor cells would selectively grow. TIA is considered as one of the appropriate systems to satisfy this requirement.

When the preculture was applied in the TIA, we were able to attain higher rates of evaluability for various types of tumors. Although the percentage of inhibition of cell proliferation to various drugs tended to give higher sensitivities by application of the preculture, in vitro responses, based on the cutoff lines as described previously, did not change significantly. Among a variety of tumors, there may be subsets which grow as nonadherent floating cells. However, the removal of the nonadherent cells did not change not only the cellular composition but also in vitro sensitivities to various drugs. In vitro/vivo correlations in 16 patients demonstrated that TIA with the preculture had a high overall predictive accuracy (92.3%), with a prediction accuracy for sensitivity of 83.3% and a resistance of 95.5%. These findings suggested that TIA with the preculture attained a sufficiently high evaluability to determine application for cancer chemotherapy while keeping high prediction accuracies.

Thus, we suggest that application of the preculture using a collagen matrix might be useful for applying TIA to broader clinical chemotherapy. Clinical benefits of the preculture for TIA should be proved by further studies which involve a number of patients with various solid tumors.

REFERENCES


Improvement of in vitro chemosensitivity assay for human solid tumors by application of a preculture using collagen matrix.

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