Characterization and the Clinical Application of Cultured Human Pulmonary Carcinoma Cells

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KOBAYASHI, S. and FUJIMURA, S. *Characterization and the Clinical Application of Cultured Human Pulmonary Carcinoma Cells.* Tohoku J. Exp. Med., 1992, 168 (2), 375-386 — We had developed a new method for the selective cultivation of cancer cells in short-term. As a result of these improvement in the culture technique, long-term subcultures of cancer cells are possible in about 80% of cases of small cell carcinoma of the lung and nearly 40% of cases of non-small cell carcinoma of the lung. 23 small cell lung carcinoma (SCLC) cell lines, 48 non-SCLC cell lines and 4 metastatic lung tumor cell lines were established in our institute using the culture method. Fractional culture of cells exhibiting the same growth pattern in primary culture produce several subtype cell lines, which can be used in experimental studies of the heterogeneity of lung cancer and in treatment of patients with lung cancer. Using subcultured cancer cells of the second or third generation, we have developed and have clinically utilized a simple sensitivity test with a Terasaki's microplate for anticancer drugs. In 15 surgical patients with SCLC treated between April 1982 and March 1985, the sensitivity test was used to select optimal anticancer drugs for postoperative chemotherapy. The routine use of the sensitivity test in selecting postoperative chemotherapy definitely improved the 3-year survival rate from 38% to 52%. ——— tissue culture; lung cancer; heterogeneity; sensitivity test

The clinical application of cancer cells requires the complete elimination of fibroblasts as early as possible to permit selective cultivation of cancer cells alone. We developed a technique for short-term selective cultivation of lung cancer cells more than 10 years ago (Kobayashi 1979). Using cultured lung cancer cells obtained by this technique, we have been performing various types of experimental and clinical studies. Our technique is based on absence of contact inhibition of cancer cell and the intensity of cell-to-cell adhesion and cell adhesion to the basal surface. As a result of these improvement in the culture technique, long-term subcultures and the establishment of cell lines are possible in about 80% of cases of small cell carcinoma of the lung (Kobayashi et al. 1989a) and nearly 40% of cases of non-small cell carcinoma of the lung (Kobayashi et al. 1990). For 1 year between March 1986 and March 1987, 18 pulmonary carcinoma cell lines

comprising 7 squamous, 4 adenocarcinoma, 3 adenosquamous, 3 small cell and 1 large cell anaplastic cell line and a pulmonary metastatic cell line derived from colon carcinoma were established in long term passages of more than 3 years (Kobayashi et al. 1990). We have now established cell lines from 48 patients

Fig. 1. Growth characteristics of SCLC cell lines ($\times 50$).

A, epithelial ; B, floating colony ; C, D, neurocyte type.

with non-small cell carcinoma, 23 with small cell carcinoma and four with metastatic lung tumors, a total of 75 cell lines.

Characteristics of cultured lung cancer cells

Lung cancer cells grow in various ways, but small cell carcinoma of the lung exhibits characteristic behavior. 82% of the cancers in primary culture displayed floating colony-type growth (Kobayashi et al. 1989a). As long-term subculture proceeded, these cells gradually adhered to the basal surface. The growth characteristics of established small cell carcinoma cell lines can be roughly divided into three types, epithelial, neurocyte and floating colony types (Fig. 1). The manner of growth of non-small cell carcinomas differed so much according to histological type and degree of differentiation that it was impossible to classify them (Fig. 2). Growth in vitro differed depending on the duration of subculture and the culture conditions. The doubling time of the established cells in serum media ranged from 0.9 to 10 days. Transplantation of such cell lines to nude mice facilitates examination of its in vivo growth and histopathological characteristics. Tumors

Fig. 2. Growth characteristics of non-SCLC cell lines ($\times 50$).

A, poorly differentiated adenocarcinoma ; B, poorly differentiated squamous carcinoma ; C, adenosquamous carcinoma ; D, large cell carcinoma.

produced by transplanting these cell lines to nude mice exhibited little formation of stroma and tended toward poor differentiation, but resembled the primary tumors in terms of histopathological profile.

Tumor cell heterogeneity in the lung cancer

Selective culture of individual groups of cells exhibiting the same growth pattern in primary culture produces several subtype cell lines, which can be used in experimental studies of the heterogeneity of lung cancer. When pulmonary small cell carcinoma cells exhibiting the floating colony type of growth were incubated in vincristine (VCR) containing medium, the number of cells adhering

Fig. 3. Morphologic changes of SCLC cells as a result of the contact with VCR.

- A) Variant clones which appeared after contact with VCR medium showing the surface attachment ($\times 50$).
- B) Original floating colony type cells ($\times 50$).
- C) Drug-affected variant cells ($\times 50$).

Fig. 4. Histopathological profiles of the primary tumor and the transplanted tumor.

A) Histopathology of the primary tumor revealed a small cell carcinoma.

B) The histologic appearance of transplanted tumor of variant clones revealed a large cell carcinoma. H.E. ($\times 100$).

to the surface increased rapidly. Fractional subculture of these cells produced two sub-cell lines, the original floating colony type cell line free from the effect of the drug and a drug-affected variant cell line growing in monolayer form (Fig. 3). Fig. 4 shows histopathological findings of the primary tumor resected at operation and the growing tumor transplanted into a nude mouse of variants showing the basal surface attachment after exposure with VCR. The histologic appearance of

Fig. 5. Four subtype cell lines with different manners of growth ($\times 50$).
A, 88-2T ; B, 88-2 ; C, 88-2FA ; D, 88-2F.

Fig. 6. Histologic findings of transplanted tumors of 4 subtype cell lines. H.E.
($\times 100$)
A, 88-2T ; B, 88-2 ; C, 88-2FA ; D, 88-2F.

transplanted tumor of variants revealed a large cell carcinoma, probably because part of the primary tumor tissue contained large cell carcinoma-like cells (Kobayashi et al. 1989a). Heterogeneity of non small cell carcinoma of the lung was also observed. Fractional culture of cells from resected primary tumor tissue of a patient with adenocarcinoma resulted in the establishment of four subtype cell lines with different manners of growth (Fig. 5). These sub-lines were transplanted to nude mice. Histopathologically, the line 88-2T cells from nude mice were small, and the line 88-2 and 88-2F cells were fairly large. Line 88-2FA consisted of characteristic signet-ring cells alone (Fig. 6). Analysis of DNA-ploidy revealed that line 88-2T was diploid, and that DNA aneuploidy was increasingly greater in lines 88-2, 88-2FA and 88-2F (Fig. 7). There were also differences in oncogenes, with line 88-2F alone exhibiting marked amplification of *c-myc*. Selective culture of lung cancer cells seems to permit determination of the heterogeneity of this cancer, which can be used in treatment.

Application to in vitro sensitivity tests

Cancer cells in primary culture, which includes cells in various sub-type cells,

Fig. 7. DNA-ploidy of 4 subtype cell lines.

Analysis of DNA-ploidy revealed that line 88-2T was diploid, and that DNA aneuploidy was increasingly greater in lines 88-2, 88-2FA and 88-2F. PBL, peripheral blood lymphocyte.

are the ideal type of cultured cancer cells for use in treatment of lung cancer. Using subcultured cancer cells of the 2nd or 3rd-generation, we have developed and have clinically utilized a simple sensitivity test for anticancer drugs (Kobayashi et al. 1987a). An advantage of this test is that it readily identifies the sensitivity of cancers to multiple drugs at multiple concentrations. Fig. 8 shows the screening test plates with which we have experimented the sensitivity of 2 lung carcinoma cells to 9 anticancer drugs. A cell suspension containing 2×10^5 cells/ml was added to each well of the Terasaki's microplate. After contact with each drug medium, the each well was washed and incubated for 10 days and stained with May-Giemsa solution or 0.1% p-iodonitrotetrazolium violet (INT) solution based on the thiazolyl blue tetrazolium bromide (MTT) assay (Mossman 1983). As shown in Fig. 8, UA1 carcinoma cells, which exhibited marked sensitivity to 5FU are slightly damaged by VCR in contrast to UA2 carcinoma cells.

Fig. 8. Screening test of 2 lung cancer cell lines (A, UA1; B, UA2) for the sensitivity to 9 anticancer drugs.

Fig. 9. Sensitivity of 16 lung carcinoma cell lines to 2 anticancer drugs. A, methotrexate; B, vincristine.

Fig. 9 shows the screening test plates with which we have examined the sensitivity of 16 lung carcinoma cell lines to 2 anticancer drugs. In addition, since sub-cultured cancer cells are used, various clinical-model experiments can be performed reproducibly. We have been clinically utilizing this sensitivity test for anticancer drugs. In 15 surgical patients with small cell carcinoma treated between April 1982 and March 1985, the sensitivity test was used to select optimal anticancer drugs for postoperative chemotherapy. The routine use of the sensitivity test in selecting postoperative chemotherapy definitely improved the 3-year survival rate from 38% to 52% (Kobayashi et al. 1987b). It is obvious that the concurrent use of effective drugs is essential to the surgical treatment of small cell carcinoma of the lung. In treating stage III small cell carcinoma of the lung, we now perform neoadjuvant therapy consisting of preoperative chemotherapy, surgery and postoperative chemotherapy with drugs selected by using the sensitivity

test on residual tumor cells. During culture of residual tumor cells following chemotherapy, the growth characteristic of many cells changes to resemble that of large cells, and these sensitivity test data indicate that the cells are resistant to multiple drugs. Nevertheless, it is important to select the most effective drug for use in postoperative chemotherapy based on a sensitivity test.

Results of recent studies and future prospects

Cancer-specific immunotherapy is a type of combined modality therapy using cultured cells. Cancer cells are cultured from each patient, and a mixed culture of autologous lymphocytes along with IL2-containing medium results in the proliferation of lymphocytes. Of these lymphocytes, 92% are cytotoxic T lymphocyte (CTL) positive for CD8, with an intensified cytocidal effect on autologous cancer cells (Inaba et al. 1990). The clinical application of CTL remains to be investigated. The preparation of monoclonal antibodies is facilitated by the use of cultured cancer cells, a possibility that we intend to examine. Hormonal therapy is an interesting approach to cancer treatment, and we have examined the feasibility of applying it to the treatment of lung cancer. Estrogen (E_2) and Progesteron (PG) receptors in the tissue of a tumor which had enlarged after transplantation of a cell line to nude mice have been examined. Using a cutoff value of 5 fmol/mg protein, 4 of 13 tumors were positive for the E_2 receptor, with detection of a high level of PG receptors in one of them (Kobayashi et al. 1989b). Using nude mice, we are in the process of investigating whether or not hormonal therapy exerts a therapeutic effect. Cancer cells are believed to secrete various markers and physiologically active substances (Sorenson et al. 1981). We measured tumor marker levels in the supernatants of cultures of 72 cell lines we had established. The positive rates were well correlated with clinical data, as shown in Fig. 10. Immunohistological analysis of tumors produced by transplanting tumor marker-producing cell lines to nude mice also reveals the state of tumor marker production. Furthermore, the use of a cell line producing high

Fig. 10. Neuron-specific enolase (ng/ml) in culture media of 72 cell lines.

Fig. 11. Cell lines producing high concentrations of tumor markers ($\times 100$).

A : 77-3 (AdSq) 147 M in culture.

CEA 25 ng/ml, CA19-9 88 u/ml, SLX >224 u/ml.

B : 87-13 (Ad) 47 M in culture.

CEA 410 ng/ml, CA19-9 39,000 u/ml, SLX >224 u/ml, SCC 7.3 ng/ml.

C : 79-1 (Teratocarcinoma) 140 M in culture.

CEA 42 ng/ml, CA19-9 250 u/ml

SLX >224 u/ml, SCC 2.1 ng/ml,

NSE 13 ng/ml.

D : 88-3 (Ad) 42 M in culture.

CEA 52 ng/ml, CA19-9 79 u/ml,

SLX 56.5 u/ml.

CEA, carcinoembryonic antigen ; CA 19-9, carbohydrate antigen 19-9 ;

SLX, sialyl Xi antigen ; SCC, squamous cell carcinoma related antigen.

concentrations of tumor markers, such as cell lines shown in Fig. 11, permits experimental examination of relationships among between sensitivity to anticancer drugs, radiosensitivity and tumor marker production. This is one of the areas we hope to pursue in future studies. Cancer cells are believed to produce various physiologically active substances. We measured the level of ACTH in the supernatant of cultured tumor cells obtained from a patient with carcinoid tumor and exhibiting Cushing's syndrome, and found it to be as high as $1,640 \mu\text{g/ml}$. The use of a large number of cultured cells enables us to study the production of various ectopic hormones cytologically. Physiologically active substances can be determined more precisely if cells are cultured in a serum-free medium. Moreover, analysis of the supernatant of serum-free medium results in detection of new physiologically active substances produced by the cancer cells, which is one of the themes we are focusing on.

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