Establishment of a Human Pancreatic Cancer Cell Line and Detection of Pancreatic Cancer Associated Antigen

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KOBARI, M., MATSUNO, S., SATO, T., KAN, M. and TACHIBANA, T. Establishment of a Human Pancreatic Cancer Cell Line and Detection of Pancreatic Cancer Associated Antigen. Tohoku J. exp. Med., 1984, 143 (1), 33-46 — We succeeded in an establishment of a human pancreatic cancer cell line (PK-1) from liver metastasis of pancreatic cancer. Primary pancreatic cancer cells grew as islands surrounded by fibroblastic cells. However, these fibroblastic cells were gradually omitted by the polygonal shaped cancer cells. This cell line contained neither zymogen granules nor trypsin indicating that this pancreatic cancer originated from pancreatic duct cells. Modal chromosome numbers of this cell line were 42 and 72 and the doubling time was 48 hr. This cell line was transplantable in athymic nude mice to form progressive tumors which had histology similar to that of the original cancer (papillotubular adenocarcinoma). Neither AFP nor ferritin but CEA was detected on the surface and in the cytoplasm of this cell line in indirect immunofluorescence. Rabbit antiserum against this pancreatic cancer cell line detected pancreatic cancer associated antigen besides CEA in the culture supernatant. This antiserum reacted with sera from patients with pancreatic cancer to form a distinct precipitin line in agarose gel which fused with the precipitin line formed between the culture supernatant of this cell line and the antiserum.

It is generally difficult to establish a continuous tumor cell line from a human adenocarcinoma of gastrointestinal organ origin (Owens et al. 1976). Above all, fewer cases have been reported about human pancreatic carcinoma cell lines of pancreatic duct origin (Lieber et al. 1975; Akagi and Kimoto 1977; Yunis et al.)

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Abbreviations used in this paper: AFP, alpha feto-protein; CEA, carcino-embryonic antigen; PBS, phosphate-buffered saline; MEM, minimum essential medium; EDTA, ethylenediaminetetraacetate; LDH, lactic dehydrogenase; AL-P, alkaline phosphatase; G6PD, glucose-6-phosphate dehydrogenase; FITC, fluorescein isothiocyanate.
because of difficulty in obtaining pancreatic cancer tissue, hardness of tumor tissue itself, and contaminating fibroblasts. In this paper, we report an establishment of continuous tumor cell line derived from a liver metastasis of human pancreatic carcinoma of the body of pancreas and also a preliminary examination of its morphological and immunological characteristics.

MATERIALS AND METHODS

Primary tumor tissue
Primary tumor tissue used to initiate a culture was obtained at operation from a liver metastasis of carcinoma of the body of pancreas because it was extremely difficult to get even a small tissue stick from a primary lesion of pancreatic carcinoma. It was found that a large tumor mass originated from the body and tail of the pancreas extensively invaded surrounding tissues and organs including the stomach, mesenterium and omentum, and was fixed firmly to the periaortic area. Ascitic fluid retention, lymph node metastases, and liver metastases were also noticed.

Method of culture
Tumor tissue completely removed from liver parenchym was washed thoroughly with PBS to eliminate residual blood and then minced with a scalpel into pieces 1 mm in diameter. The tumor pieces were minced with scissors to smaller pieces which thereafter were washed through \# 30 mesh with MEM (Nissui Co., Ltd., Tokyo) supplemented with 20\% fetal bovine serum into single cells or cell clusters. After standing this cell suspension for several minutes, all precipitates were collected and resuspended in MEM supplemented with 20\% fetal bovine serum at a relatively high cellular concentration. Then each 0.5 ml of cell suspension was distributed into wells of a small plastic 4 well multi dish (Nunc, Denmark). The incubation was carried out at 37°C in 5% CO₂ in air. Medium was changed every 3 days and serial transfer of primary and established cultures was carried out by treatment with 0.25\% trypsin (GIBCO, Grand Island, N.Y., USA) in 0.02\% EDTA-PBS after confluent monolayers were obtained.

Cytology
Monolayer cells on coverslips were fixed in 10\% formaldehyde solution and then stained with May-Giemsa dye or periodic acid-Schiff reagent.

Growth curve
1 X 10⁵ cells were cultured on 35 mm plastic plates (Falcon Plastics, Oxnard, Calif., USA) in 1.5 ml MEM supplemented with 10\% fetal bovine serum. Cell numbers were counted in triplicate plates everyday without exchange of medium.

Serum requirement
1 X 10⁵ cells were seeded on 35 mm plastic plates in MEM supplemented with serial concentrations of fetal bovine serum from 0.1 X 10⁴ to 10 X 10⁴ and were cultured in 1.5 ml MEM supplemented with 10\% fetal bovine serum on 35 mm plastic plates. The cell numbers were counted on day 3.

Chromosome analysis
Cells treated with colchicine (40 \(\mu\)g/ml) for 20 hr were harvested and treated with 0.9\% sodium citrate solution for 8 min, then were fixed in a 2 : 1 mixture of absolute methanol and glacial acetic acid and expanded on a coverslip by frame. Chromosome counts were
Performed after Giemsa staining.

**Enzyme assay**

After 7 days incubation in serum free medium, the spent culture supernatant was collected and concentrated 10-fold for assay of enzymes secreted into the medium. Intra-cellular enzyme assay was performed using sonication material of $2 \times 10^6$ cells. Trypsin, LDH, AL-P, and G6PD were assayed in cooperation with Ohotsuka Assay Laboratories.

**Immunological examination**

1. **Implantation in athymic nude mice (BALB/C nu/nu).** $5 \times 10^6$ cells were harvested and washed with PBS and then implanted into 3 athymic nude mice subcutaneously.

2. **Immunofluorescence examination.** Indirect immunofluorescence staining of tumor cells was performed using rabbit anti-AFP antiserum, anti-ferritin antiserum (Hoechst Behring Institute Marburg-Lahn, FRG), and rabbit anti-CEA antiserum (DAKO-immunoglobulins, Ltd., Denmark). After 30 min incubation at $37^\circ C$, cells were washed and stained with FITC-labeled goat anti-rabbit IgG (Hoechst Behring Institute Marburg-Lahn, FRG) for 30 min at $37^\circ C$ and observed under a fluorescence microscope.

3. **CEA assay.** Radioimmunooassay of spent culture medium for CEA was performed by employing Dainabot Kit (Dainabot, Tokyo) and 10-fold concentrated spent culture medium was examined by a micro-Ouchterlony method.

4. **Preparation of anti-PK-1 cells antiserum and the specificity study.** $5 \times 10^6$ cells in 1 ml PBS were emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into the back of the rabbit 5 times at weekly intervals and 2 weeks after the last immunization anti-PK-1 cells antiserum was obtained. The specificity of this antiserum was examined by a micro-Ouchterlony method for crude CEA preparation, standard AFP preparation (Hoechst Behring Institute Marburg-Lahn, FRG), fetal liver ferritin, and normal human sera or sera of patients with malignancy.

**RESULTS**

Histological feature of the liver metastasis of pancreatic cancer used was a proliferation of tumor cells forming glandular structure. Each tumor cell was polymorphic and mitotic cells were observed everywhere. This tumor was diagnosed as papillo-tubular adenocarcinoma of large duct origin (Fig. 1). Though single cells in the primary culture did not show any tendency to adhere to the bottom of dish, cell clusters or small pieces of tissues well attached to the dish. After 5 days of cultivation, fibroblastic cells were observed around them. Two weeks later, the bottom of the dish was covered with fibroblasts which surrounded polygonal epithelial cells aggregated like islands. In order to remove the fibroblasts, floating fibroblasts were aspirated after 10 min treatment with 0.25% trypsin in 0.02% EDTA-PBS and then, island-like tumor cell colonies remained were cultured. After repeating this treatment, tumor cell colonies being contaminated with fairly little fibroblasts were transferred to new dishes and finally epithelial tumor cells without any fibroblasts could be cultured (Fig. 2). Morphological appearance of cultured cells was characterized by polymorphic large cells with a large nucleus having several nucleoli. Though many mitoses were observed, no tubular arrangement of cultured cells was visible (Fig. 3). In PAS staining, strongly stained cells mixed with many unstained cells were
observed (Fig. 4). In electron microscopy, the cells were closely joined with each other through interdigitation-like projections. The nucleus was irregular in shape, and rich in heterochromatin. In cytoplasm, many mitochondria and large vacuoles having granular mucus were visible but no zymogen granules were observed (Fig. 5). The cultures are successfully going on to transfer for up to 26 times by now. Its growth rate is relatively slow with population doubling time

Fig. 1. Microscopic appearance of liver metastasis of pancreatic cancer from which PK-1 cell line was originated.

Fig. 2. Phase contrast microscopic appearance of PK-1 cells showing a confluent pavement-like monolayer of polygonal epithelial cells.
of 48 hr reaching to a plateau level of growth until 5 days of culture (Fig. 6). Our PK-1 cells grow well at a fetal bovine serum level over 3% in the medium but rather decrease in cell number at a serum level below 3%. It was necessary to plant cells at a number over $2 \times 10^4$ per 1.5 ml in 35 mm dish to gain sufficient growth (Fig. 7). The result of chromosome analysis of 100 metaphasic cells indicated an aneuploid pattern with two modal numbers of 42 and 72 (Fig. 8).
Immunological analysis

Transplantation tumor in athymic nude mice. In athymic nude mice receiving $5 \times 10^6$ cells, palpable tumor appeared 4 weeks after subcutaneous injection and it took 5 months for the progressive tumor of $10 \times 10$ mm in size. The tumor was serially transplantable in athymic nude mice. Histology of the tumor resembled that of liver metastatic lesion used for primary culture. Polymorphic cells were arranged in a honey comb appearance forming a tubular pattern (Fig. 9).

Immunofluorescence. No fluorescence was seen either on the cell surface or in
Fig. 7. Growth condition of PK-1 cells. Upper: Serum concentration. Lower: Cell concentration at seeding.

Fig. 8. Modal chromosome numbers of PK-1 cells.
the cytoplasm, when performed with anti-AFP antiserum or anti-ferritin antiserum. On the other hand, anti-CEA antiserum gave strongly positive results on cell surface and also weakly in cytoplasm (Fig. 10).

**CEA analysis.** The CEA level of confluent cells cultured for a week without medium change was increased to 52.7 ng/ml or 105 ng/10⁶ cells. By the micro-Ouchterlony method of culture supernatant concentrated 10 times, a clear precipi-
Establishment of Human Pancreatic Cancer Cell Line

Establ...tion line was visible with anti-CEA antiserum but not with anti-AFP antiserum or anti-ferritin antiserum (Fig. 11).

Analysis of anti-PK-1 cell antiserum. By the micro-Ouchterlony method, this antiserum formed one precipitin line with sera from patients with pancreatic cancer in addition to a common precipitin line with normal human sera. This specific line with sera from patients with pancreatic cancer was not observed with sera from patients with gastric or colon cancer examined. Further, it was different from AFP or ferritin, and was not detected with anti-CEA antiserum (Fig. 12a, b, c). In addition, this antiserum formed one distinct precipitin line with the culture supernatant of PK-1 cell. This precipitin line fused with the precipitin line formed with serum from patients with pancreatic cancer and was different from the precipitin line formed by CEA and its antiserum (Fig. 13).

Discussion

It is very valuable to establish human pancreatic cancer cell lines for fundamental and clinical studies of pancreatic cancer cells. Especially, biological, oncological, and immunological characterizations of the cells are of importance for
diagnostic and therapeutic aim of pancreatic cancer.

As for establishing cultured tumor cell lines, it is in general difficult to establish them from biopsy specimens of the primary tumor site of the gastrointestinal organ, because of the fewness of biopsy sticks, low tumor cell density, mixing of normal epithelial cells and fibroblasts, and sometimes hardness of the primary tumor lesion (Giard et al. 1973; Owens et al. 1976; Machida et al. 1977). Therefore, many cancer cell lines have been established from floating cells of ascites or pleural effusions and metastatic lesions (Akagi et al. 1977; Morgan et al. 1980; Chen et al. 1982). The use of ascites or pleural effusions diminishes the number of contaminating normal epithelial cells in cultivation, but increases the
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 Establishment of Human Pancreatic Cancer Cell Line

frequency for collection of a sufficient number of cancer cells. On the other hand, much more cancer cells without mixing of normal epithelial cells can be obtained from metastatic lesions than from primary lesions and it is easier to get biopsy sticks from liver metastasis than from the primary lesion, especially in the case of pancreatic cancer. Cancer cell populations in metastatic sites are apt to adapt to the in vitro environment than those in primary tumor sites.

From this situation, we attempted to establish a pancreatic cancer cell line from liver metastasis of cancer of the body of pancreas and succeeded in it. There may be, however, some differences in cell population between primary lesion and metastatic lesion, e.g. the differences in differentiation, antigen expression or invasiveness (Fidler 1975; Morgan et al. 1980; Bosslet and Schirrmacher 1982).

Some characteristics of the established pancreatic cancer cell lines are summarized in Table 1. The cultured cancer cells derived from ductal papillo-tubular adenocarcinoma in the pancreas grew at first as an epithelial cell colony surrounded by a fibroblast monolayer, and then became a confluent cell layer after repeating removals of fibroblasts with trypsin treatment in succeeded cell transfers. Thus, our success may be partially due to feeder effect of fibroblasts in primary culture as suggested by others (Aaronson et al. 1970; Rheinwald and Green 1975).

Microscopically, PAS positive cells piled up here and there and many large cells with several nucleoli or in mitoses were observed. Electron microscopically, the cells contacted each other via interdigitation-like projections. Many vacuoles containing mucus were observed in the cells, but no zymogen granules were seen.
The culture cells did not secrete nor contain any trypsin activity, but contained intracellular enzymes such as G6PD and AL-P. From these results, the origin of our cell line is considered to be pancreatic duct epithelium capable of mucus secretion.

Heterotransplantation tumors in athymic nude mice of this cancer cell line showed histological features similar to those of the original tumor. Therefore, the tumors produced in athymic mice are also very useful for studies on chemotherapy of pancreatic cancer and analysis of pancreatic tumor associated antigen as a tumor marker. The CEA level of the spent culture medium increased from 0.5 ng/ml before cultivation up to 52.7 ng/ml after 7 day cultivation. The presence of CEA was also observed intensively on the plasma membrane and in the cytoplasm of the established pancreatic cells by indirect immunofluorescence. This is compatible with the result of the paper on established pancreatic cancer cell lines described by Akagi and Kimoto. (1977). Thus, all above mentioned characteristics of the cultured cells indicate that they originate from pancreatic duct carcinoma, but not from acinar cell carcinoma (Kaku et al. 1980). Although most of other established pancreatic cancer cell lines reported so far originated from undifferentiated or poorly differentiated carcinoma, our cell line seemed to be derived from fairly well differentiated one. In our case, the period required for the establishment of cell line was fairly short without lag phase. So, it is possible that our cell line may preserve many characteristics of the original pancreatic

<table>
<thead>
<tr>
<th>Reporters</th>
<th>Cancer site</th>
<th>Starting material</th>
<th>Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kobari et al.</td>
<td>Body &amp; tail</td>
<td>Liver metastasis biopsy</td>
<td>Papillotubular adenocarcinoma</td>
</tr>
<tr>
<td>Chen et al. 1982</td>
<td>Head</td>
<td>Ascites</td>
<td>Moderately differentiated adenocarcinoma</td>
</tr>
<tr>
<td>Akagi and Kimoto 1977</td>
<td>Head &amp; body</td>
<td>Ascites</td>
<td>Undifferentiated anaplastic carcinoma</td>
</tr>
<tr>
<td>Yunis et al. 1977</td>
<td>Body &amp; tail</td>
<td>Primary tumor biopsy</td>
<td>Undifferentiated carcinoma</td>
</tr>
<tr>
<td>Lieber et al. 1975</td>
<td>Head</td>
<td>Resected specimen</td>
<td>Undifferentiated carcinoma</td>
</tr>
<tr>
<td>Grant et al. 1979</td>
<td>Head</td>
<td>Resected specimen</td>
<td>Anaplastic and poorly differentiated carcinoma</td>
</tr>
<tr>
<td>Morgan et al. 1980</td>
<td>Head</td>
<td>Lymph node metastasis</td>
<td>Well differentiated adenocarcinoma</td>
</tr>
</tbody>
</table>
Establishment of Human Pancreatic Cancer Cell Line

Next, we tried to detect pancreatic cancer associated antigen. Rabbits were immunized with the established pancreatic cancer cells (PK-1) to obtain antiserum specific to PK-1 cells. Anti-PK-1 antiserum specifically reacted with PK-1 cells and sera from patients with pancreatic cancer, but also contained antibodies reactive to a normal serum component and CEA. After absorbing this antiserum with a CEA-producing gastric cancer cell line (KATO-3), the antiserum still retained specificity to PK-1 cells, but no longer to the CEA-positive gastric cancer cell line detected by indirect immunofluorescence (data not shown). In preliminary experiments, this pancreatic cancer associated antigen was undoubtedly different from AFP, ferritin, besides CEA. However, it is not certain whether the antigen is identical with either of pancreatic cancer associated antigens described previously (Banwo et al. 1974; Gelder et al. 1978; Schultz and Yunis 1979).

### References


