Establishment and Characterization of Human Pancreatic Cancer Cell Lines in Tissue Culture and in Nude Mice

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Three human pancreatic cancer cell lines, designated as KP-1N, KP-2 and KP-3 have been established in both tissue cultures and in nude mice. The KP-1N and KP-3 tumors were obtained from liver metastases of pancreatic tumors and the KP-2 tumor was of primary pancreatic origin. The patients' tumors from which KP-1N and KP-2 were derived showed characteristics of adenocarcinoma, and the KP-3 tumor had adenosquamous carcinoma characteristics. Inoculations of samples from surgical specimens into athymic nude mice resulted in tumor formation, with the tumors histologically closely resembling the original neoplasms. Subcutaneous injections of the established cell lines also induced tumor formation and the tumors histologically resembled the original lesion in the cases of KP-2 and KP-3 tumors, but the KP-1N tumors in the mice were histologically different from the surgical specimen. The KP-1N, KP-2 and KP-3 cell lines have been cultured continuously in a medium supplemented with 10% fetal calf serum for more than 20, 21 and 17 months, respectively. The KP-2 and KP-3 cell lines produced and released carbohydrate antigen 19-9 into the spent medium but the KP-1N cell line did not. KP-1N and KP-3, produced liver metastases after intrasplenic injection into nude mice, whereas KP-2 produced few liver colonies. Cell lines highly metastatic to the liver, KP-1NLs and KP-3Ls, were isolated from the liver colonies of KP-1N and KP-3, respectively.

Key words: Pancreatic tumor cell line — Liver metastasis of pancreatic tumor — Pancreatic tumor cell establishment

Carcinomas of the pancreas are one of the most frequent causes of cancer deaths in Japan, and the incidence is increasing.1) Biological models are necessary for the study of pancreatic cancer, in that little is known about the biology, chemotherapy, or metastasis of this neoplasm. Thus, permanent cell lines and xenografts of human pancreatic cancers could provide information on the biological properties and therapeutic response of this tumor that could lead to effective approaches for treatment of this disease.

To date, attempts to establish such lines have been limited with only a few successful cases, including PANC-1,2) HGC-25,3) MIA PaCa-2,4) QGP-1,5) COLO 357,6) CAPAN-1,7) ASPC-1,8) RWP-1,9) RWP-2,9) HPL-Y1,10) T2M-4,11) PSN-1,12) SUIT-2,13) and MDAPanc-3.14)

Liver metastasis is very often observed in human pancreatic tumors even in early stages. Recently, an attempt to demonstrate metastatic activity in the liver from colon tumors has been carried out using intrasplenic (i.e.) injections of cells.15,16) It is necessary to study an experimental model system to determine whether pancreatic tumor cells metastasize to the liver.

In this paper, the establishment and metastatic activity of three new cell lines derived from human pancreatic cancers of ductal origin is reported.

MATERIALS AND METHODS

KP-1N, KP-1NLs Tissue was obtained surgically from a 69-year-old Japanese man. Histologically, the specimen was classified as a liver metastasis from moderately differentiated tubular adenocarcinoma of the pancreas. A cell line was established from the resulting tumor in the spleen of a nude mouse (Balg/C/nu/nu; CLEA Japan, Osaka) into which a human liver metastatic tumor cell suspension was injected. The cell line was designated as KP-1N. KP-1NLs was established from the resulting tumor nodules in the liver of mouse, into the spleen of which KP-1N cells had been injected.

KP-2, KP-2N The tissue originated from a primary biopsy taken from a 65-year-old Japanese woman. It was classified as moderately differentiated tubular ad-
enocarcinoma of pancreatic ductal origin. The cell line was established from the tissue and designated as KP-2. KP-2N cell line was obtained from a xenograft in a nude mouse into which the tumor mentioned above had been subcutaneously inoculated. KP-3, KP-3N, KP-3Ls KP-3 was obtained from an autopsy specimen of liver metastasis from a pancreatic tumor of a 75-year-old Japanese man. The tissue was classified as a metastasis of adenosquamous carcinoma of the pancreatic duct. KP-3N was established from a human tumor xenograft in a nude mouse. KP-3Ls was established from the resulting tumor nodules in the liver of mouse, into the spleen of which KP-3 cells had been injected.  

**Primary culture**  
The tumor specimens were washed in Hanks' balanced salt solution (HBSS) containing penicillin (100 U/ml) and streptomycin (100 μg/ml) (GIBCO, Grand Island, NY). The tumor tissue was minced into small pieces and dispersed with 0.1% collagenase (Wako Pure Chemical, Osaka), 0.005% DNase I (Sigma, St. Louis, MO) and 0.002% hyaluronidase (Sigma) in Daigo's T medium (Nihon Sepiyaku Co. Ltd., Tokyo). After exposure to this enzyme solution for 30 min at 37°C, the cell suspension was centrifuged at 250g for 5 min. The cell pellet was suspended in 5 ml of 2 mM EDTA in Ca²⁺ and Mg²⁺-free phosphate-buffered saline solution [PBS(−)]. The cell suspension was incubated for 15 min at 37°C and then centrifuged again in the same manner. The cell pellet was resuspended in 0.2% collagenase in Daigo's T medium and incubated for 30 min at 37°C. The cell suspension was filtered through a mesh and centrifuged. The cell pellet was suspended in Daigo's T medium supplemented with 10% fetal calf serum (FCS) (Hazleton Research Products, Inc., Lenexa, KS), penicillin (100 U/ml), and streptomycin (100 μg/ml) and seeded into Petri dishes (Corning/Iwaki Glass, Tokyo). Collagen-coated Petri dishes were used for KP-3. A half volume of the medium was exchanged every 3 to 7 days. The elimination of fibroblastic cells was accomplished chemically by brief exposure to 0.05% trypsin and 0.1% EDTA in PBS(−) and mechanically by scrubbing with a silicon rubber policeman. This procedure was repeated until no further fibroblastic cell growth could be observed. About 1 to 2 months later, epithelioid cells without contaminating fibroblastic cells had propagated to a confluent stage in the Petri dishes. The cells were then passaged by treatment with 0.25% trypsin and 0.1% EDTA in PBS(−).  

**Determination of the doubling time in vitro culture**  
A suspension of 3 × 10⁴ cells was plated into 60 mm Petri dishes with Daigo's T medium supplemented with 10% FCS, and the number of cells was counted at intervals of 24 h for 6 days. The cell lines used for the doubling time determination were KP-1N (passage 11 and 20), KP-1NLs (highly metastatic cell line selected from KP-1N), KP-2 (passage 16), KP-2N (passage 10), KP-3 (passage 13) and KP-3N (passage 11). The determination was done with three dishes for each cell line.  

**Chromosome analysis**  
Chromosomes were examined in exponentially growing cells. Karyotypes were analyzed by the use of trypsin-G banding. The cell lines used for the analysis were KP-1N (passage 40), KP-2 (passage 20), and KP-3 (passage 15).  

**Determination of tumor markers**  
Carcinoembryonic antigen (CEA), carbohydrate antigen (CA) 19-9, and sialyl SSEA-1 antigen (SLX) levels in the spent medium were determined by radioimmunoassay. The assay was done at Otsuka Assay Laboratories Ltd., Tokushima.  

**Transplantation of human tumor tissue or cultured cell lines into nude mice**  
Pieces of the original neoplasms or 1 × 10⁷ cultured cells were inoculated subcutaneously into nude mice that were 6 to 8 weeks old. The resulting tumors were measured with calipers and their volume was estimated by using the following formula; V = L × W × H/2 (V, volume; L, length; W, width; H, height). The cell lines used for the experiment were KP-1N (passage 35), KP-2 (passage 18) and KP-3 (passage 16). When a tumor diameter reached 10−12 mm, the tumor was excised for serial transplantation and histological examination, and for the establishment of the KP-2N and KP-3N cell lines. The tumor growth rate was determined using three nude mice for each cell line.  

**Histopathology**  
All the tumor tissues were examined histologically. The xenografts were fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned on a microtome, and stained with hematoxylin and eosin.  

**Liver metastasis assay of the newly established cells**  
The capacity to form tumors in the liver was studied following an i.s. injection as described by Fidler et al. Briefly, nude mice were anesthetized with sodium pentobarbital (Pitman-Moore, Washington Crossing, NJ) and their left flanks were prepared for sterile surgery. A small cutaneous incision was made in the left flank and carried down through the peritoneal wall. Viable tumor cells (1 × 10⁶/0.05 ml in HBSS) were injected into the spleen with a 27-gauge needle. The spleen was then returned to the peritoneal cavity, and the abdominal wall was closed with sutures. The mice were killed approximately 90 days after the injection, and autopsies were performed. Liver tumor lesions were harvested and established in culture as individual cell lines. The cell line established from KP-1N nodule was designated as KP-1NLs and the cell line from KP-3 nodule was designated as KP-3Ls. The cells from in vitro passages 5−10 were injected into the spleens of nude mice, and the ability to produce metastases in nude mice was determined.
RESULTS

Establishment of cultured cell lines and in vitro growth characteristics Three permanent cell lines, one from a xenograft in a nude mouse spleen, and two from patients, were established. The cell lines, designated KP-1N, KP-2, and KP-3, have been continuously cultured for more than 20, 21 and 17 months, respectively. KP-2N and KP-3N cell lines were also established from subcutaneous xenografts in nude mice.

The KP-1N, KP-2 and KP-3 cells grown on plastic surfaces coated for tissue culture had typical epithelial morphologies (Fig. 1). The KP-1N cells were characterized by the occasional appearance of a vacuolated cytoplasm and by two morphologies: an elongated form and a polygonal form. A basophilic cytoplasm and giant cells containing a single nucleus or multiple large nuclei were seen sporadically in KP-2 cells. Monolayers of KP-3 cells contained large, polygonal cells with a clear, basophilic cytoplasm. Collagen-coated dishes were necessary for the KP-3 cells in the primary culture stages, but after five passages they were no longer necessary.

During the exponential phase, the population doubling times of KP-1N (passages 11 and 20), KP-2 (passage 16), and KP-3 (passage 13) were estimated to be 24.0 h, 43.2 h, and 28.8 h, respectively (Fig. 2A). The doubling times of KP-2N (passage 10) and KP-3N (passage 11) established from xenografts in nude mice were estimated to be 38.4 h and 24.0 h, respectively. The doubling time of KP-1NLs was 21.6 h.

The chromosome counts of metaphase cells revealed the modal peak to be 58 for KP-1N, 142 for KP-2, and 68 for KP-3 (Fig. 3). Chromosome analyses of KP-1N, KP-2, and KP-3 cell lines indicated that they were human tumor cell lines with many abnormal chromosomes.

Tumor growth in nude mice The KP-1N, KP-2, and KP-3 cell lines were all tumorigenic and almost 100% takes were obtained in nude mice. Inoculations with $1 \times 10^7$ KP-1N or KP-3 cells in nude mice resulted in detectable tumors after 7–10 days. Nude mice, given an injection of $1 \times 10^7$ KP-2 cells, developed tumors after 4–6 weeks. The KP-1N (passage 40), KP-2 (passage 18), and KP-3 (passage 16) tumors grew exponentially in nude mice with volume doubling times of 5 days, 23 days, and 16 days, respectively (Fig. 2B). When the tumor diameters reached 10–12 mm, the tumors were excised for histological examination (Fig. 4). Microscopically, KP-2 or KP-3 tumors exhibited characteristics similar to those of the original neoplasms. It was histologically found that the KP-1N tumors were poorly differentiated adenocarcinoma and different from the original tumor, which was a moderately differentiated adenocarcinoma.

Production of tumor markers The levels of some tumor markers were checked in the spent medium after 24 h of incubation of the confluent-state cells, because these markers were found in all of the patients' sera (Table I). No tumor marker proteins were detected in the spent medium of KP-1N (passage 5 and 37). Small amounts of CEA and SLX and a rather high level of CA 19-9 were found in the spent medium of KP-2 (passage 5). KP-2 cells were still producing tumor marker proteins at pas-

Fig. 1. Photomicrographs of KP series cells growing in monolayer culture. Phase-contrast optics, ×300 (original magnification). (a), KP-1N (passage 3); (b), KP-2 (passage 3); (c), KP-3 (passage 3).
Fig. 2. Growth curves of KP series cells. A, In vitro growth curves; B, growth curves in nude mice; ●, KP-1N; △, KP-2; ▲, KP-2N; □, KP-3; ■, KP-3N.

Fig. 3. Histograms of chromosome numbers. Fifty metaphases for KP-1N (passage 40), 32 metaphases for KP-2 (passage 20) and 20 metaphases for KP-3 (passage 15) were analyzed.

sage 27. KP-2N possessed marker synthetic activity at passage 23. In the serum of the KP-2 tumor-bearing mice, a high level of CA 19-9, and low levels of SLX and CEA were detected. The levels of CA 19-9 and SLX in the KP-3 (passage 4) incubation medium and in the serum of the tumor-bearing nude mice were as high as those of the patients, but the markers disappeared from the incubation medium of KP-3 cells at passage 14. The tumor marker level in the KP-3N medium was similar to that of KP-3.

Liver metastatic colonies in nude mice The capacity of the cells from the three cell lines to produce tumor growth in the liver of nude mice was examined, subsequent to injection of cells into the spleen. Some metastatic colonies were observed in the liver of nude mice after an i.s. injection of KP-1N (passage 20) (Table II). The colonies were less angiogenic than were the liver metastatic colonies from other pancreatic tumor cell lines. Liver metastatic colonies were also found on the liver edges after an i.s. injection of KP-3 cells (passage 5) (Table II). The KP-1NLs and KP-3Ls cell lines were established from liver colonies. They were highly metastatic to the liver of nude mice, when KP-1NLs or KP-3NLs was injected into spleens of nude mice (Table II). When KP-2 or KP-2N (both passage 20) cells were injected into the spleen of nude mice, no liver metastases were detected after 3 months. Thus, some cell clones with metastatic activity could be found in KP-2 cells.
DISCUSSION

In vitro establishment of cancer cells, particularly pancreatic cancer cells, is often difficult because of their low propagation ability and contamination by rapidly growing fibroblasts. Now three human pancreatic cancer cell lines have been successfully established in cultures and in nude mice. The resulting tumors that were induced in mice were histologically similar to the human tumor tissues which were collected during surgery or at autopsy.

KP-2N and KP-3N established from xenografts in nude mice proliferated with shorter doubling times than those of KP-2 and KP-3 (Fig. 2A), but the biological and biochemical characteristics of KP-2N and KP-3N, such as the morphology of the cells and tumor marker production, were approximately the same as those of KP-2 and KP-3, respectively (Table I).

The i.s. injections of the tumor cells were used to check the activity of liver metastases, because the liver is the most frequent metastatic target of pancreatic tumors. The number of tumors observed in the liver may depend on both the tumor cell affinity for mouse liver and the cell
Table I. The Levels of Three Marker Proteins, CEA, CA 19-9 and SLX, in Spent Medium of Each Cell Line

| Cell lines | CEA\(^a\) | CA 19-9\(^b\) | SLX\(^c\) |
|------------|-----------|--------------|-----------|
| KP-1N      |           |              |           |
| passage 5  | ND        | ND           | ND        |
| passage 37 | ND        | ND           | ND        |
| KP-2       |           |              |           |
| passage 4  | 0.21      | 91.6         | 2.1       |
| passage 27 | 0.27      | 97.8         | 2.1       |
| KP-2N      |           |              |           |
| passage 10 | 0.17      | 90           | ND        |
| passage 23 | 0.09      | 101.6        | ND        |
| KP-3       |           |              |           |
| passage 2  | ND        | 171.2        | 6.9       |
| passage 14 | ND        | 0.8          | ND        |
| KP-3N      |           |              |           |
| passage 4  | ND        | 140.6        | 11.1      |
| passage 18 | 1.8       | 3.2          |           |

\(a\) Values are ng/1 x 10^6 cells/24 h.
\(b\) Values are U/1 x 10^6 cells/24 h.
ND, not detected.

Table II. Tumorigenic Activity of KP-series Cells in Livers of Nude Mice after Intrasplenic Injections

| Cells     | Days after inoculation | Tumor-bearing mice/Number of mice used | Number of colonies in liver |
|-----------|------------------------|----------------------------------------|----------------------------|
| KP-1N     | 78                     | 1/3                                    | 25                         |
|           | 94                     | 1/3                                    | uncountable                |
| KP-1NLs   | 37                     | 3/3                                    | 2, 6, uncountable          |
|           | 47                     | 3/3                                    | 38, 42, 33                 |
| KP-2      | 90                     | 0/3                                    |                            |
| KP-2N     | 90                     | 0/2                                    |                            |
| KP-3      | 91                     | 2/2                                    | 2, 5                       |
| KP-3N     | 45                     | 0/2                                    |                            |
| KP-3Ls    | 56                     | 1/1                                    | uncountable                |
|           | 105                    | 1/1                                    | uncountable                |

growth rate in the liver. KP-1N and KP-3 resulted in several colonies 3 months after the injection, but no visible KP-2N or KP-2 colonies could be found in the liver 3 months after the injection. KP-1N and KP-3 cells grew faster than KP-2 cells in in vitro cultures, with doubling times of 1.8-fold and 1.5-fold, respectively (Fig. 2B). From these results, the differences of the metastatic activity of the cell lines could be explained by the cell growth rate in the liver. However, KP-1NLs produced many more tumor colonies than KP-1N in 1 month, in spite of having approximately the same doubling times. KP-1NLs cells might have a high affinity for the liver. The KP-2 cell line is not likely to contain a cell population with an affinity for the liver, but there may be some in KP-1N and KP-3 cells. KP-1N (passage 20) produced more metastatic colonies in the liver of nude mice after an i.s. injection than earlier passaged KP-1N did (data not shown). The liver affinity of KP-1N cells might change during in vitro passage. The liver affinity may be one of the expressive phenomena of tumor cell progression.

KP-1N and KP-3 cell lines were established from the liver metastatic regions of pancreatic tumors and KP-2, from a primary tumor of the pancreas. The difference in metastatic ability may have resulted from the tissues of origin of the tumors.\(^15\)

The KP-2, KP-3, and KP-1N cell lines had 142, 68, and 58 chromosomes, respectively. It is not thought that the metastatic activity of the cells in nude mice and the growth activity correlate with only the chromosome number of the cell. Rearrangement and mutation of genes may be responsible for the malignancy of the tumor cells. Point mutations of cell DNA have often been observed in tumors of the pancreas.\(^16, 19\) Ki-ras DNA point mutations on codon 12 of exon 2 have been found in KP-1N and KP-3 DNA (GGT→GAT on KP-1N and GGT→GTT on KP-3), but not in KP-2 DNA. There may be some correlation between metastatic activity and DNA mutation. The cell lines established in the study were relatively well characterized as regards the metastatic activity. No human pancreatic tumor cell line with well characterized metastatic activity has previously been reported. Cell lines such as KP-1N, KP-1NLs, KP-3 and KP-3Ls may be useful not only for studying the mechanism of pancreas tumor metastasis but also for antimetastatic drug evaluation.

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