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Establishment and Characterization of a New Human Pancreatic Cancer Cell Line, YPK-1

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Abstract A new human pancreatic cancer cell line, designated YPK-1, was established from ascites collected from a 62-year-old Japanese man. This cell line has been maintained *in vitro* for 96 months with stable growth through 260 passages. We characterized the YPK-1 cell line morphology, doubling time, levels of tumor markers secreted into culture medium, chromosomal content, and tumorigenicity in nude mice. YPK-1 cells have a clear polygonal-shaped cytoplasm, round nuclei, and prominent nucleoli. The cells grew as an adherent monolayer and lacked contact inhibition. The doubling time at the tenth passage was 49.3 hours. YPK-1 cells secreted detectable amounts of carcinoembryonic antigen, carbohydrate antigen 19-9, carbohydrate antigen 125, carbohydrate antigen 50, DU-PAN-2, and SPan-1. Chromosome analysis revealed that the diploid chromosome number ranged from 57 to 62 with a mode of 61. Tumorigenicity was demonstrated by development of tumors after subcutaneous injection of YPK-1 cells into nude mice. We believe that this cell line will contribute to studies of the biological and molecular properties of pancreatic carcinoma and to the development of therapeutic strategies for this tumor.

Introduction

Pancreatic carcinoma is a disease with a poor prognosis and is considered by many to be one of the deadliest malignancies[1)]. Although many therapeutic strategies have been developed, most have not appreciably improved the prognosis of patients with pancreatic carcinoma, which has an overall 5-year survival rate of less than 5%[2)]. Therefore, it is important to establish human pancreatic cancer cell lines as *in vitro* models for studying the biological and molecular properties of this tumor. During the past 20 years, establishment of many human pancreatic cancer cell lines has been reported [2-25)]. These cell lines have provided valuable information about various aspects of pancreatic cancer, including carcinogenesis, histogenesis, diagnosis, and therapy. Because pancreatic carcinoma remains a disease with a generally poor prognosis [1)], more cell lines are needed to investigate this neoplasm in greater depth.

In our laboratory, six esophageal cancer cell lines have been established [26,27)], and these cell lines have provided some valuable information about tumor markers [28)]

and the biological and molecular properties of esophageal cancer [29–31]. Since 1994, we have attempted to establish additional pancreatic cancer cell lines based on our experience with cell culture techniques. This paper reports the establishment and characterization of a new human pancreatic cancer cell line that we have designated YPK-1.

Materials and methods

Patient

A 62-year-old Japanese man was admitted to our hospital on August 23, 1994, because of epigastralgia and a weight loss of 15 kg over 6 months. He was diagnosed as having pancreatic cancer with multiple liver metastases. On admission, his serum levels of tumor markers were 27.1 ng/ml in carcinoembryonic antigen (CEA), 54963 IU/ml in carbohydrate antigen (CA) 19-9, 156 IU/ml in CA125, 13000 IU/ml in DU-PAN-2, and 5600 IU/ml in SPan-1. He received irradiation therapy. Ascites appeared, and cytologic examination with Papanicolaou, Giemsa, alcian blue, and periodic acid Schiff (PAS) staining was performed. Adenocarcinoma cells were seen frequently (Fig. 1) and were positive for PAS and alcian blue. The patient died 2 months after admission.

Culture medium

Dulbecco's modified Eagle's medium (DMEM) (Nissui, Tokyo, Japan) supplemented with 100 U/ml of penicillin ,100 μg/ml of streptomycin (GIBCO BRL, Rockville, MD), 12 mM sodium bicarbonate, and 20% fetal calf serum (FCS) (M.A. Bioproducts, Walkersville, MD) was used as the primary culture medium (PCM). DMEM containing 10% FCS was used as the maintenance culture medium.

Culture procedure

Collected serous ascites was placed in tissue culture flasks with a plug seal screw cap (25 cm^2 style; Becton Dickinson, San Jose, CA) at 37℃ in an atmosphere of 5% CO_2 in air. After tumor cells adhered to the bottom of the flasks and formed nests, the cells were washed in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) (Nissui) and cultured in PCM at 37℃ in an atmosphere of 5% CO_2 in air. The medium was changed three times each week. For subculture, cells were detached by treatment with trypsin-EDTA (0.05% trypsin, 0.53 mM ethylenediamine tetraacetic acid Na_4; GIBCO BRL). The cells were observed

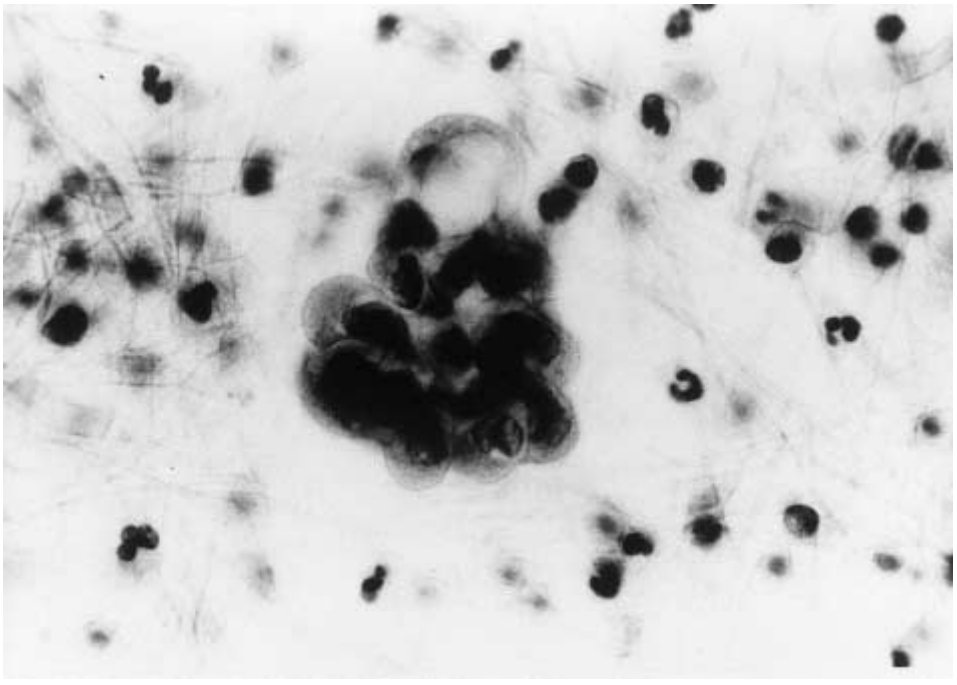

Fig. 1. Cytologic appearance of the original tumor cells in ascites (Papanicolaou, original magnification, x100).

daily with a phase-contrast microscope (Nikon, Tokyo, Japan).

Growth curve

Growth curve was obtained by seeding the cells at $1x10^5$ cells per tissue culture dish (60 x 15 mm style; Becton Dickinson) in 5 ml of DMEM containing 10% FCS in duplicate. The medium was changed every 2 days after seeding. The cells were detached with trypsin-EDTA, and the average number of viable cells from two dishes was determined by counting cells that excluded trypan blue with a Bürker-Türk hemocytometer. The doubling time of the cell population was estimated from the logarithmic growth phase.

Tumor markers

Secretion of tumor markers was assessed in cells grown for 48 hours in 5 ml of DMEM containing 10% FCS. Supernatants were obtained by centrifugation at 3000 rpm for 10 minutes at 4℃, and assayed for CEA, CA 19-9, CA 125, CA 50, DU-PAN-2, and SPan-1 with the use of commercially available assay kits (CEA, CEA RIABEAD, Dainabot, Tokyo, Japan; CA 19-9, Centocor CA19-9 RIA Kit, TFB, Tokyo, Japan; CA125, Centocor CA125II IRMA Kit, TFB; CA50, TR-FIA Kit, Pharmacia, Tokyo, Japan; DU-PAN-2, Determiner DU-PAN-2, Kyowa, Tokyo, Japan; SPan-1, SPan-1 RIABEAD, Dainabot).

Chromosome study

Three days after seeding the cells were placed into 25 cm^2 style tissue culture flasks, colcemid (0.04 μg/ml) was added to the flasks, and cells were incubated at the 37℃ for 2 to 4 hours. Cells were detached with 0.25% trypsin and treated with hypotonic 0.075 M KCl solution for 10 minutes. They were then centrifuged at 3000 rpm for 10 minutes at 4℃, and fixed with a mixture of methanol and acetic acid (3:1) twice. Cells suspended in fixative were dropped onto slides, dried over a flame, and stained with Giemsa. G-banding was carried out by staining the cells with Giemsa after 5 minutes of treatment with 0.0125% trypsin [26].

Heterologous transplantation

Five- to 6-week-old male athymic nude mice (Balb/c *nu/nu* mice; Japan SCL, Hamamatsu, Japan) were used to examine the tumorigenicity of YPK-1. Cells ($1x10^6$) were suspended in 100 μl of PBS and injected subcutaneously into the abdomens of mice. The mice were killed 4 weeks after inoculation, and the subcutaneous tumors were processed for light microscopic examination, immunohistochemical staining, and electron microscopic examination.

Light microscopic examination

For light microscopic observation, formalin-fixed paraffin blocks of specimens were sectioned at 3 μm. Sections were dewaxed, rehydrated, and stained with hematoxylin-eosin. For immunohistochemical staining, sections were dewaxed, rehydrated, and stained with antibody to CEA (Kyowa Medicus Co., Tokyo, Japan), CA 19-9 (TFB), and CA 125 (TFB) and an avidin-biotin complex immunoperoxidase technique (DAKO LSAB kit; DAKO Co., Carpinteria, CA) as described below. Briefly, nonspecific binding was blocked by incubation of sections with non-immune goat serum diluted in 0.05 M Tris/HCl buffer, pH 7.6, containing 6% carrier protein and 15 mM sodium azide as preservative. Samples were incubated with primary antibody (diluted 1:100 in PBS) for 30 minutes, incubated in secondary antibody (diluted 1:100 in PBS) for 15 minutes, and then incubated in 1:400 streptavidin for 15 minutes at room temperature. Peroxidase activity was detected with 3,3'-diaminobenzidine as a chromogen with H_2O_2 as a substrate. Samples were counterstained with hematoxylin and mounted. As negative controls, samples were stained as above but with PBS substituted for the primary antibody.

Electron microscopic examination

For electron microscopic observation, specimens were fixed in Karnovsky's fixing fluid for 1 hour at room temperature. After rinsing three times with 0.1 M sodium cacodylate buffer, samples were postfixed in 2% osmium tetraoxide with Millonig's buffer (1:1), dehydrated in a graded series

of ethanols, and embedded in Epon 812. Specimens were sectioned on an ultramicrotome, stained with uranyl acetate and lead citrate, and examined under a Hitachi H-500 electron microscope.

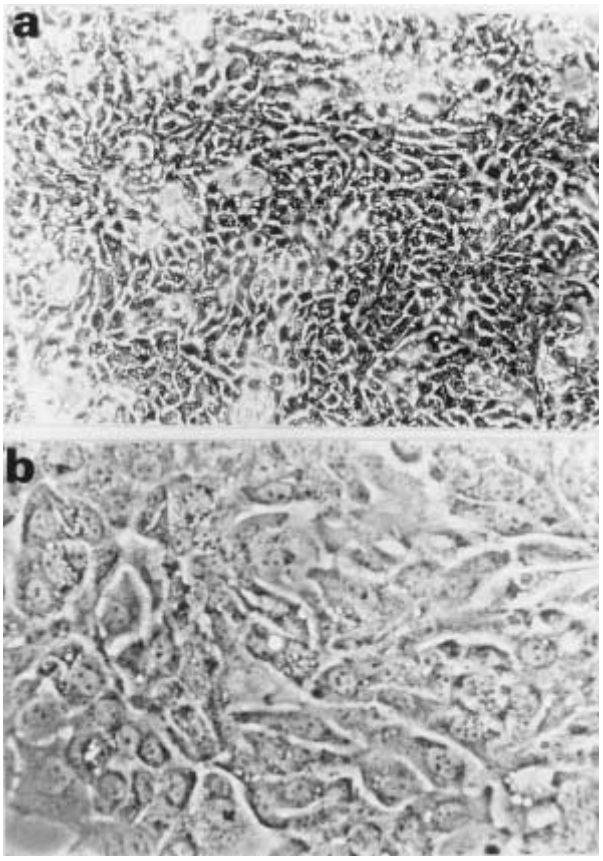

Fig. 2. Phase contrast microscopic appearance of YPK-1 cells (original magnification, (a) x100 (b) x400).

Mycoplasma detection

After the cells were passed twice through antibiotic-free medium, the medium to be tested was exposed to the cells for 3 days. Mycoplasma contamination was examined by Gen-Probe Mycoplasma T.C. Rapid Detection Kit (Gen-Probe Co., Inc. San Diego, CA).

Results

Establishment and morphology of the YPK-1 cell line

Serous ascites was collected and placed in 25 cm^2 style tissue culture flasks. A few days after primary culture, small colonies of epithelial-like cells were observed in several flasks. The ascites was then removed, and PCM was added. These colonies increased in size, and piling up of the cells was observed in the center of the colonies. When the epithelial-like cells reached semi-confluence at 14 days after primary culture, cells were subcultured into two 25 cm^2 style tissue culture flasks and maintained in DMEM containing 10% FCS. Thereafter, subcultures were performed periodically. Growth of fibroblast-like cells, which were easily distinguished from epithelial-like cells under phase con-

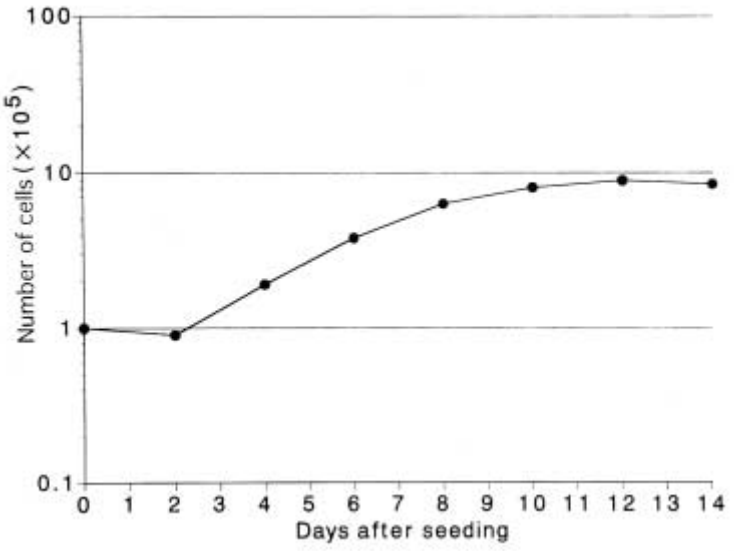

Fig. 3. Growth curve of YPK-1 cells at the tenth passage.

trast microscopy, was also observed around the colonies. The growth of the epithelial-like cells appeared to be inhibited or limited by the overgrowth of the fibroblast-like cells. The fibroblast-like cells were removed by differential trypsinization and serial passages. The resulting cell line grew continuously and was passaged for 260 generations over 96 months. It has been designated YPK-1.

YPK-1 cells were found to proliferate in a pavement-like arrangement. After reaching confluence, the cells had a tendency to pile up and showed lack of contact inhibition. These cells were shown to have pleomorphic features, being primarily polygonal-to-spindle shaped with eosinophilic cytoplasm and oval-to-round nuclei (Fig. 2). Occasionally, multinucleated giant cells were observed.

Growth curve

The growth curve was obtained by seeding YPK-1 cells at the tenth passage. After a lag phase of 48 hours, cells entered a rapid logarithmic growth phase. The doubling time of the cell population in the logarithmic phase was 49.3 hours (Fig.3).

Tumor markers

Tumor markers CEA, CA 19-9, CA 125, CA 50, DU-PAN-2, and SPan-1 were detected in culture media. The secretion of tumor markers paralleled the growth curve in all phases (Table 1). CEA, CA 19-9, CA 125, CA 50, DU-PAN-2, and

Table1 Levels of tumor markers secreted by YPK-1 cells

Days after seeding	CEA(ng/ml)	CA19-9(IU/ml)	CA125(IU/ml)	CA50(IU/ml)	DU-PAN-2(IU/ml)	SPan-1(IU/ml)
0	0	0	0	0	0	0
2	9.2	930	8	300	230	220
4	5.5	600	10	220	100	190
6	13	1,700	34	590	340	510
8	38	4,400	78	1,600	1,500	960
10	190	16,000	170	3,800	9,100	3,000
12	410	17,000	200	7,100	21,000	3,800
14	610	23,000	250	8,000	26,000	4,700

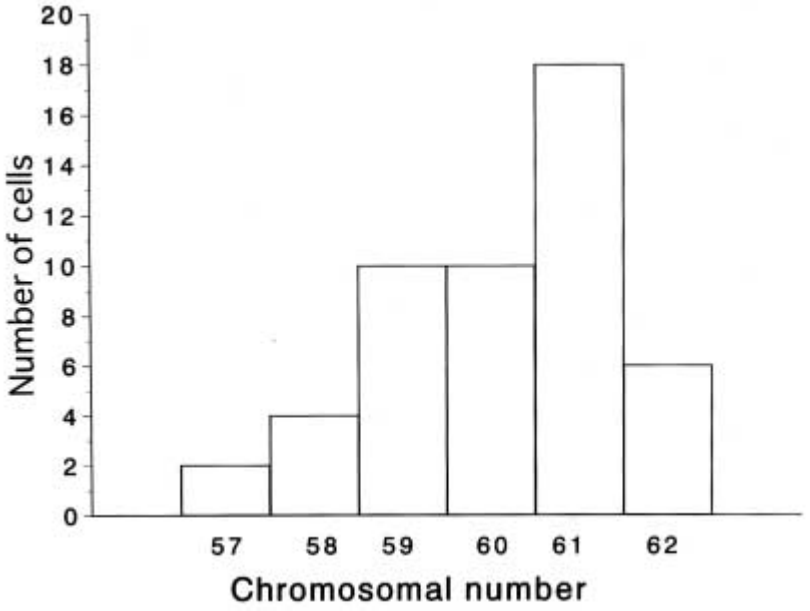

Fig. 4.Distribution of chromosomal number at the twelfth passage.

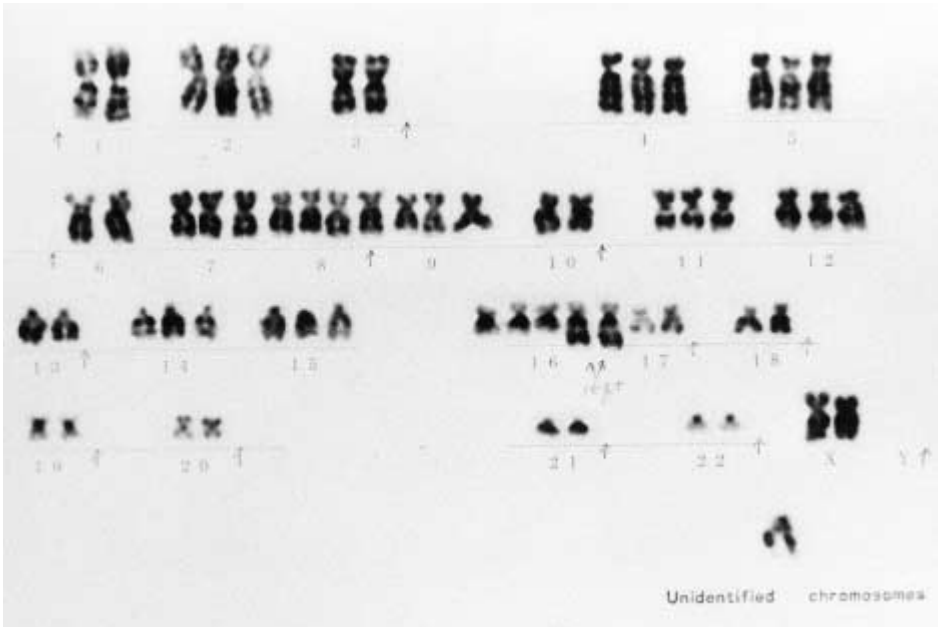

Fig.5. A G-band karyotype of YPK-1 cells at the twelfth passage. Note that there are 61 chromosomes.

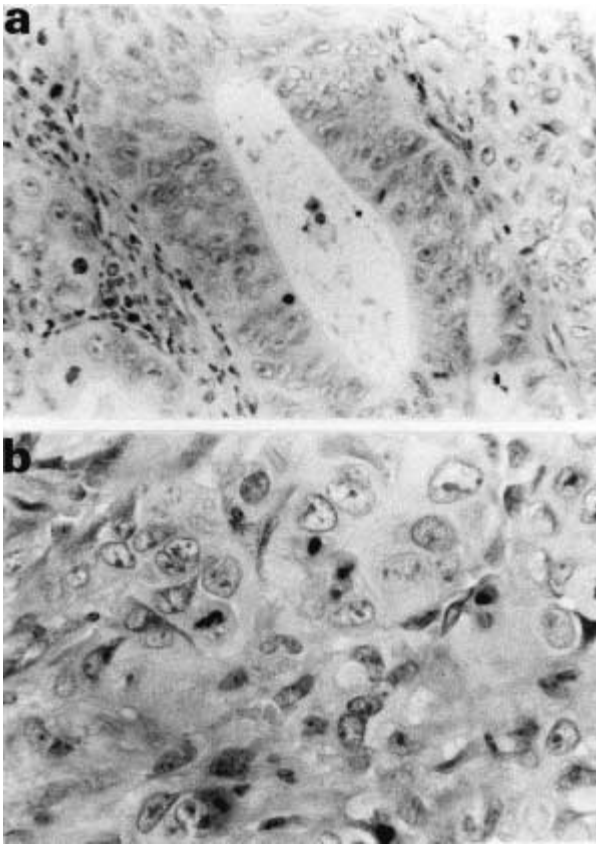

Fig.6. Histological appearance of the subcutaneous tumor in nude mice. (Hematoxylin-Eosin, original magnification, (a) x100 (b) x400).

SPan-1 were not detected in maintenance culture medium.

Chromosome study

YPK-1 cells at the twelfth passage were used for chromosome analyses. The diploid number of chromosomes ranged from 57 to 62, with a mode of 61 (Fig.4). G-band analysis of cells containing 61 chromosomes revealed a karyotype of XX, +2, +4, +5, +7, +8x2, +9, +11, +12, +14, +15, +16, add(16)(qter)x2, +mar (Fig.5).

Heterologous transplantation and morphology of tumor cells transplanted into nude mice

YPK-1 cells at the ninth passage were suspended in PBS and injected subcutaneously into the abdomens of nude mice. Visible subcutaneous tumors were detected approximately 18 days after inoculation and gradually increased in size. The subcutaneous tumors reached their greatest dimension (10 mm) by 28 days after inoculation, but metastatic foci in the internal organs were not observed.

Under light microscopic examination, tumors showed the histological features of moderately differentiated tubular adenocarcinoma (Fig.6). The tumor cells had eosinophilic, columnar or polygonal cytoplasm, and round-to-oval nuclei (Fig. 6a). Thin bundles of collagen fibers were present in the stroma (Fig.6b). Expression of CEA, CA 19-9, and CA 125 in the

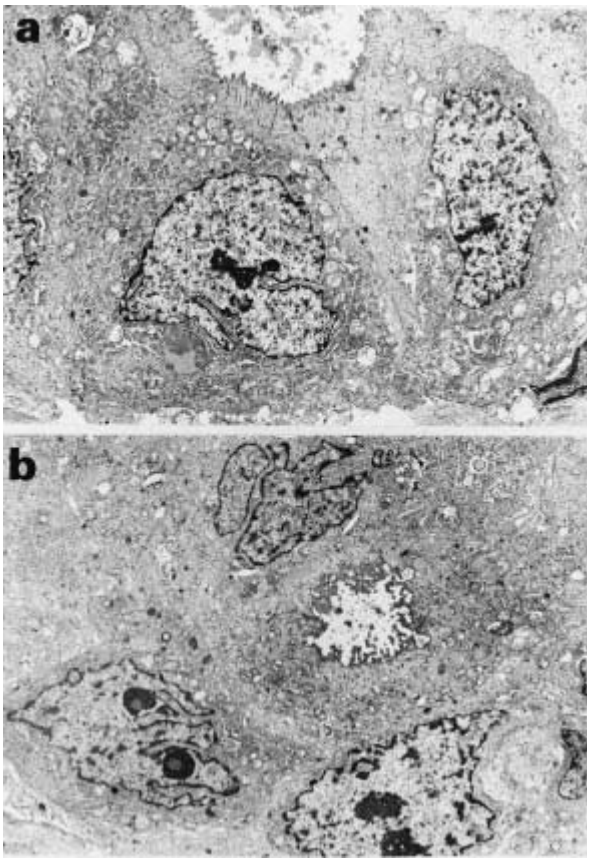

Fig.7.Electron microscopic appearance of the subcutaneous tumor in nude mice. (original magnification, (a) x6000 (b) x5500).

cytoplasm of subcutaneous tumors was detected by immunohistochemical staining (data not shown).

Under electron microscopic examination, the tumor cells had microvilli at the apices of the cells (Fig.7a). In the cytoplasm, organelles were poorly developed, and no zymogen or endocrine granules were observed (Fig.7b).

Mycoplasma detection

Mycoplasma contamination was not present.

Discussion

Since the first report of transplantation of a human colon cancer into nude mice by Rygaard and Povlsen in 1969 [32], transplantation of human malignant tumors into nude mice has been reported by many authors [26]. Because transplanted tumors retain the morphological and functional characteristics of the original tumor, transplantation into nude mice is recognized as a useful *in vivo* tumor model. Moreover, the chances of obtaining fresh cancer cells are increased if the transplanted tumors develop. Therefore we subcutaneously transplanted human esophageal cancer cells into nude mice to establish tumor cell lines. The overall success rate for transplantation of esophageal cancers is 83% in our laboratory[26]. Six human esophageal cancer cell lines have been established from subcutaneously transplanted cells[26,27]. However, this method is complicated and expensive. In the present study, we attempted to establish cell lines by use of an easy method in which ascites was placed in tissue culture flasks. Using ascites as a culture material has several advantages, including no or less contamination by normal epithelial cells and fibroblasts, ease of obtaining many free cancer cells, repeatability of cultivation [6], and lack of damage because tumor cells are obtained without the use of enzymes. We established the YPK-1 cell line with this method.

YPK-1 cells were characterized as human pancreatic cancer cells on the basis of the following observations. Morphologically, YPK-1 cells exhibit an epithelial cell growth pattern with a pavement-like arrangement and show a lack of contact inhibition. YPK-1 cells had stable growth for more than 96 months (260 passages), and tumorigenicity was shown by the development of tumors after subcutaneous injection of YPK-1 cells into nude mice. Light and electron microscopic examination of tumors transplanted into nude mice showed that these cells had the features of adenocarcinoma cells. We confirmed that these cells were of human origin by chromosome analyses, and we concluded that the cell line established was derived from a human pancreatic cancer.

Recently, many tumor markers have been used for diagnosis and management of malignant tumors. Tumor markers have five potential uses: screening, diagnosis, establishing prognosis, monitoring treatment, and detecting relapse[33]. For

pancreatic cancer, CA19-9 and CEA are useful tumor markers[34,35]. YPK-1 cells secret detectable amounts of not only CEA and CA 19-9 but also CA125, CA50, DU-PAN-2, and SPan-1. Serum levels of these tumor markers in our patient were elevated. High serum levels of these tumor markers are sometimes found in patients with pancreatic cancer, but the mechanism of secretion remains unclear. It may be revealed by further investigation of YPK-1 cells.

With respect to morphology, doubling time, levels of tumor markers secreted into culture medium, and karyotype, there are some differences in YPK-1 cells compared with other previously established pancreatic cancer cell lines. In general, clinical courses of pancreatic cancers vary. This means that additional cell lines that have different characteristics are necessary.

In conclusion, the YPK-1 cell line is a new human pancreatic cancer cell line that should contribute to studies of the biological and molecular properties of pancreatic carcinoma and to the development of therapeutic strategies for this pancreatic cancer.

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