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—Original Article—

Establishment and characterization of a cultured cell line derived from nitrosamine-induced pancreatic ductal adenocarcinoma in Syrian golden hamsters

Seiji SAITO, Nobuyuki NISHIMURA, Yoshiki KUBOTA, Kunio YAMAZAKI, Takashi SHIBUYA and Hiroshi SASAKI

Third Department of Internal Medicine, Toyama Medical and Pharmaceutical University, Toyama 930-01, Japan

Summary: Seven kinds of pancreatic ductal adenocarcinomas induced by N-nitrosobis(2-hydroxypropil)amine in Syrian golden hamsters were established as transplantable tumor lines on syngeneic animals. These tumor lines were all well or moderately differentiated adenocarcinomas, showing various velocities of growth, unrelated to the grade of histological differentiation. A cell line, designated HaP-T1, was established in continuous tissue culture from one of these homografts. The cells grew in a monolayered sheet with an approximately 17-hour population doubling time. Chromosomal analysis revealed that the modal chromosomal number of the cell line was 44. HaP-T1 cells showed apparent tumorigenicity both on syngeneic hamsters and athymic nude mice, but they grew much faster when injected into the former animals. Morphological characteristics of HaP-T1 cells and tumors induced by HaP-T1 inoculation in both animals revealed apparent epithelial characteristics resembling ductal adenocarcinoma of the pancreas. These transplantable tumor models will contribute to the further investigation in the field of pancreatic cancer. *Gastroenterol Jpn 1988;23:183–194*

Key Words: *Experimental pancreatic cancer, Pancreatic cancer cell line, Transplantable pancreatic cancer model*

Introduction

Pancreatic cancer is one of the most dismal cancers of the digestive organs at the present time. In recent years, there has been an increasing interest in the development of experimental models of chemically-induced pancreatic cancer in animals. In 1974, Pour[1] described an experimental model of consistent pancreatic carcinogenesis following chronic nitrosamine administration in Syrian golden hamsters. Althogh pancreatic tumors in this model resembled those found in humans in morphological and biological aspects, there have been only a few studies of established transplantable pancreatic carcinoma lines of ductal cell origin for detailed ongoing biologic investigations[2-6]. Moreover, only a very limited number of reports have been made about established hamster pancreatic cancer cell line in tissue culture[7-9]. We have developed seven kinds of homologously transplantable tumor lines derived from N-nitrosobis (2-hydroxypropyl) amine (BHP)-induced pancreatic adenocarcinomas arising in Syrian golden hamsters and have propagated a continuous cell line in tissue culture from one of these homografts. The present paper describes mor-

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Address for correspondence: Seiji Saito, M.D., Third Department of Internal Medicine, Toyama Medical and Pharmaceutical University, 2630 sugitani, Toyama-shi, Toyama 930-01, Japan.
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phological and biological characterization of this tissue cultured cell line designated HaP-T1.

Materials and Methods

Animals

The inbred GN strain Syrian golden hamsters were obtained from Nippon Institute for Biological Science (Oume, Tokyo) and propagated by sibling matings in the laboratory animal center of our university. Athymic nude mice (BALB/c, nu/nu, from Nippon Clea Co. Tokyo) were also maintained in a conventional condition.

Induction of tumor

Fifty five female golden hamsters, 8 weeks of age, were injected subcutaneously once a week with 500mg/kg body weight of BHP (Nakarai Chemical Co. Osaka) with saline as the vehicle. Five hamsters were injected with saline alone as controls. Several animals were sacrificed at every week 15 to 22 weeks after the initial BHP injection.

Homologous transplantation of the pancreatic tumors

The visible tumor nodules in the pancreas of the autopsied animals were excised and minced, and the fragments were transplanted surgically in the dorsum of syngeneic hamsters. Tumor growth was monitored by measurement with vernier calipers once a week. When tumor diameter reached about 15 to 20mm they were excised for serial transplantation and histological examination.

Cultivation in vitro

One of the homograft tissues (designated 'J', in the third passage) was excised and finely minced by scissors into small pieces under sterile conditions. The minced tumor tissue was placed into Falcon No 3001 tissue culture dishes (Falcon Plastics, Oxnard, Calif.) and 12ml of Eagle's minimal essential medium (MEM) (Nissui Seiyaku Co. Tokyo) supplemented with 10% fetal bovine serum (M.A.Bioproducts, Walkersville, Maryland), gentamycin (20mg/l, Schering Corp. Kenilwarth, NJ), nonessential amino acids for MEM (10ml/l, Flow Laboratories Inc. Rockville, MD), glutamine (0.3g/l, Nissui Seiyaku Co.) and sodium pyruvate (110mg/l, Flow Laboratories Inc.) was added into each of the dishes. The culture dishes were then incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. The culture medium was changed twice weekly. During the early weeks of primary culture the epitheloid tumor cell colonies and fibroblastoid cells grew in mixture. After the consecutive culture for about two months without passage, the mesenchymal cells stopped their growth and the epitheloid cells predominated. The cells were routinely passed with 0.25% trypsin: 0.02% EDTA solution in Dulbecco's modified phosphate buffered saline (PBS) (all from Flow Laboratories Inc.) every 3 or 4 days. At the present time the cells grow as an epitheloid monolayer without fibroblast and have undergone more than 150 serial passage. The cultured cells were free of mycoplasma contamination when tested with Hoechst Stain Kit (Flow Laboratories Inc.). We designated this cell line HaP-T1.

Growth characteristics of HaP-T1 in vitro

The cells of the 40th passage were used to determine the population doubling time and saturation density. 2×10^5 of HaP-T1 cells were placed in triplicate culture dishes (Falcon No 3003, 22cm^2). Cell numbers were determined every 24 hours for 5 days. The numbers of cells per dish at each sample time were plotted on semilog graph paper, and the cell population doubling time and the saturation density were read from the graph.

Chromosomal analysis

The cultured cells of the 40th passage in exponential growth phase were treated with 0.1μg/ml colcemid for two hours at 37°C. The cells were trypsinized and treated with hypotonic KCl solution (0.075M) for 15 minutes. The cells were fixed in methanol:acetic acid

Table 1 Incidence of pancreatic cancers in Syrian golden hamsters injected with BHP

Times of BHP injection	Number of hamsters	Number(%) of animals with pancreatic cancer		
		microscopic	macroscopic	transplantable
0 (control)	5	0 (0)	0 (0)	0 (0)
15	4	1 (25)	0 (0)	0 (0)
16	2	2 (100)	1 (50)	0 (0)
17	5	5 (100)	1 (20)	0 (0)
18	5	4 (80)	4 (80)	2 (40)
19	10	10 (100)	4 (40)	2 (20)
20	3	2 (67)	2 (67)	0 (0)
21	2	2 (100)	2 (100)	2 (100)
22	1	1 (100)	1 (100)	1 (100)

(3:1), and were dropped onto wet slides, dried and stained with Giemsa. Fifty metaphases were selected for counting chromosomal numbers.

Tumorigenicity study

Fourteen syngeneic hamsters and ten athymic nude mice were used as hosts for tumor cell inoculations. HaP-T1 cells of the 80th passage were trypsinized and the cells were suspended in saline. 0.5ml of cell suspension with density of 2×10^6/ml for each hamster and 0.2ml with density of 5×10^6/ml for each nude mouse were injected subcutaneously into their backs. The size of these HaP-T1 induced tumors was measured once a week with vernier calipers. Some of the tumor tissues were obtained for histological and ultrastructural examination.

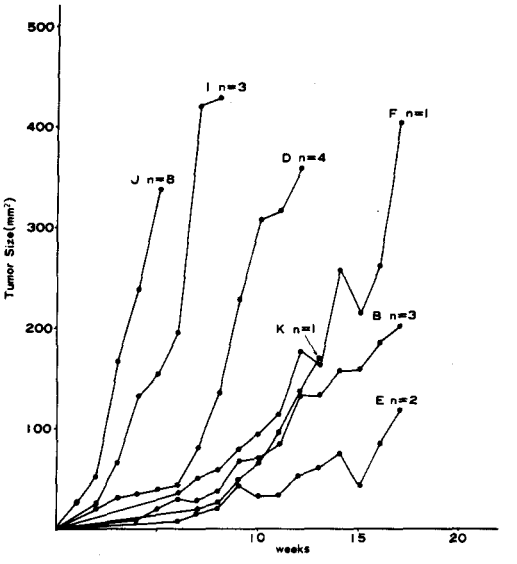

Fig. 1 Growth curves of initially transplanted pancreatic cancers on syngeneic hamsters.

Morphological study

Tumor tissue was fixed in 10% buffered formalin, embedded in paraffin and stained with hematoxilin and eosin (H-E), and alcian blue and periodic acid Shiff (AB-PAS). Tumor tissue was also minced, fixed in 4% paraformaldehyde plus 1% glutaraldehyde, postfixed with 2% osmium tetroxide, dehydrated through an ethanol series, and embedded in Epon 812. Ultrathin sections cut on an LKB-2088 ultramicrotome were stained with uranyl acetate and lead citrate, and examined with a Hitachi H-300 transmission electron microscope. HaP-T1 cells in monolayer culture were photographed by a phase contrast microscope. For electron microscopy of the monolayer cultured cells, a confluent tissue culture dish was washed once with PBS, and 4% paraformaldehyde plus 1% glutaraldehyde followed by 2% osmium tetroxide were added into the dish for fixation. Then dehydration was performed by graded ethanols. Gelatin capsules filled with Epon 812 were put on the monolayer cells upside down and polimerization was performed at 45°C for 12 hours and 60°C for 48 hours. The dish and gelatin capsules were dipped in xylene to dissolve the plastic dish. The methods for staining and observation were the same as those for the tumor tissue described above.

Table 2 Characteristics of primary tumors and transplanted tumor lines of BHP-induced pancreatic carcinoma in Syrian golden hamsters

Tumor line	Times of BHP injection	Localization of primary tumor	Diameter of primary tumor	Histology	Passage[1]
B	18	splenic lobe	5 mm	well.[2], tub.[3]	4
D	18	splenic lobe	7 mm	well., pap-tub.[4]	7
E	19	splenic lobe	5 mm	well., tub.	3
F	19	splenic lobe	5 mm	mod.[5], tub.	4
I	21	splenic lobe	8 mm	well., tub.	8
J	21	gastric lobe	10 mm	mod./well., pap-tub.	10
K	22	gastric lobe	5 mm	well., tub.	3

[1] times required for serial passage in the first year after initial transplantation.
[2] well differentiated
[3] tubular adenocarcinoma
[4] papillo-tubular adenocarcinoma
[5] moderately differentiated

Results

Tumor induction

Thirty two hamsters were sacrificed 15 to 22 weeks after the initial BHP injection. Incidence of the pancreatic tumor is summarized in Table 1. Eighty four percent of the animals (27 of 32) had microscopic and/or macroscopic pancreatic tumors at the time of autopsy. Histological examination of these tumors revealed papillary, tubular or cystic adenocarcinomas.

Morphological and biological features of the homografts

Homologous transplantation was attempted on twelve visible tumors and seven kinds of transplantable lines were successfully established. These tumor lines were designated B, D, E, I, J and K, respectively. Morphology and growth characteristics of these lines are summarized in **Table 2** and **Figure 1**. On light microscopy, these pancreatic cancer lines were all moderately to well-differentiated adenocarcinomas (**Fig. 2 a-d**). Some of them showed papillotubular form with many goblet cells (**Fig. 2a**), but most of the tumors showed tubular structures lined by columnar (**Fig. 2b,d**), cuboidal and flattened epithelial cells (**Fig. 2c**). Heterogenlity of the grade of histological differentiation was sometimes observed in the same specimen of an homografted tumor line (**Fig. 2c,d**). AB-PAS positive materials were often found in the apical cytoplasm of the epithelial cells and in the lumen (not shown in Figure). As shown in **Figure 1**, the seven tumor lines showed extremely different growing velocity. This trend did not vary with serial passage. Tumor lien 'J' showed the rapidest growth and was selected for the material of the source for tissue culture.

Morphological features of HaP-T1 cells

The appearance of HaP-T1 cells in the monolayer culture is shown in **Figure 3**. HaP-T1 cells grew as relatively compact colonies and showed a cobble stone like appearance in semiconfluent state. When cell growth became confluent the cells began to pile up and to detatch from the plastic surface. Ultrastructurally, HaP-T1 cells had a high nucleocytoplasmic ratio, abundant nuclear heterochromatin and scarce intracytoplasmic organelles. Luminar structure was often observed in the moolayer culture (**Fig. 4a**). In a higher magnification, the tumor cells showed scarce mitochondria, Golgi complexes and rough endoplasmic reticulum. Abundant free ribosomes were observed (**Fig. 4b**). Desmosomes and junctional complexes were usually observed between the adjacent cells (**Fig. 4b,c**). Cells, facing a luminal structure, had elongated and branched microvilli on a luminal cell surface and many secretory

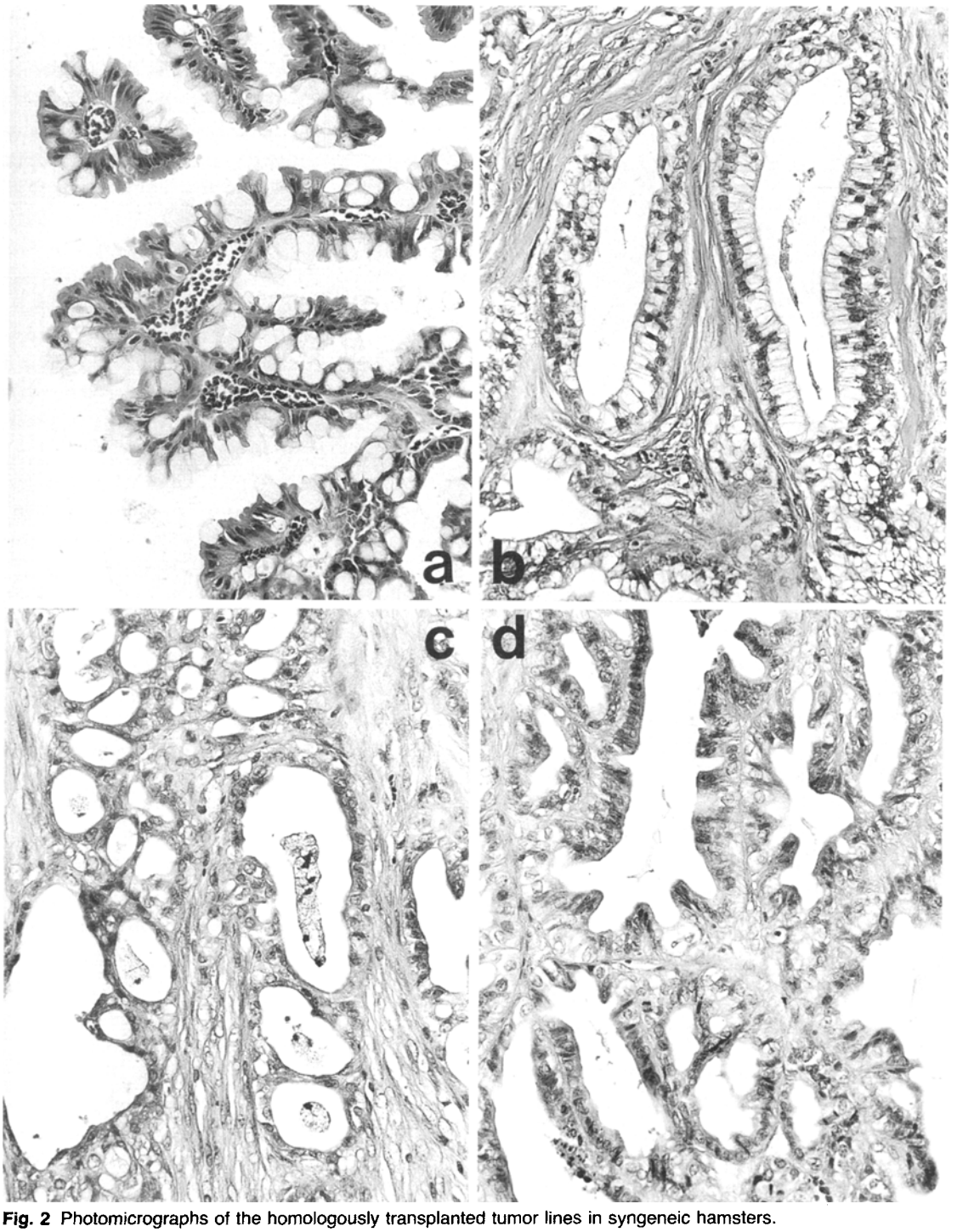

Fig. 2 Photomicrographs of the homologously transplanted tumor lines in syngeneic hamsters.
H-E stain, ×200
a: Hitological feature of the tumor line designated 'D'.
b: Histological feature of the tumor line designated 'E'.
c: Histological feature of the tumor line designated 'J'.
d: Histological feature of another part of the specimen of the tumor line 'J'.

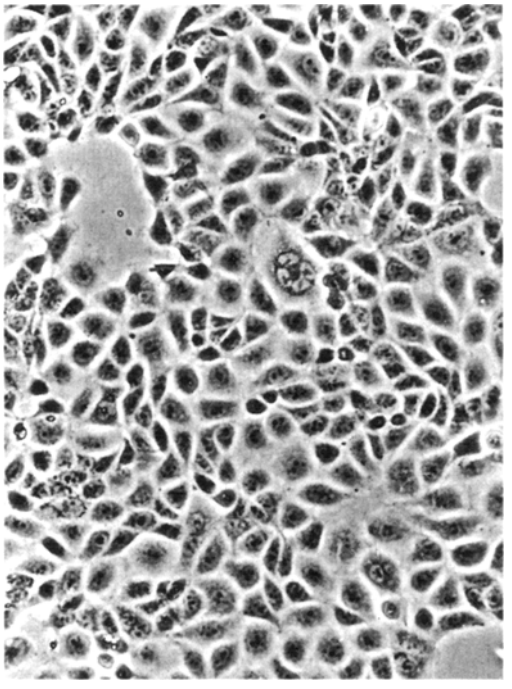

Fig 3 Phase contrast micrograph of living HaP-T1 cells in a semiconfluent monolayer culture. ×100

granule-like structures, ranging from 200 to 300 nm in diameter, in the apical cytoplasm. Abndant glycocalyx-like materials were also indicated on the microvilli and the apical plasmalemma (**Fig. 4d**).

Growth characteristics in vitro

Growth curve of the cultured HaP-T1 cells is shown in Figure 5. The population doubling time was about 16.9 hr. and the saturation density was $4.4\times10^5/cm^2$.

Chromosomal analysis

The distribution of chromosomal numbers of the HaP-T1 cells of the 40th passage is shown in **Figure 6**. The modal number was 44.

Morphology and growth characteristics of HaP-T1 induced tumors in syngeneic animals and athymic nude mice

HaP-T1 cells inoculated subcutaneously into the back of the syngeneic hamsters formed rapidly growing tumors invading adjacent muscles. The overlying skin was ulcerated about 4 weeks after the inoculation. Advanced tumors routinely penetrated the thoracic and abdominal walls and directly infiltrated the viscera, but no hematogenous metastasis were observed. HaP-T1 cells also successfully grew on athymic nude mice by subcutaneous inoculation, but growing velocity of the tumor was much slower than that on syngeneic hamsters (**Fig. 7**). Ulceration of the skin and necrosis of the tumor were occasionally observed in the xenograft, but neither direct invasion into the viscera nor distant metastasis were found in athymic mice. The histological examination with light microscopy on the homografted HaP-T1 induced tumor revealed moderately differentiated tubular adenocarcinoma essentially similar to the histology of the original homografted tumor (**Fig. 8a**). The histological appearance of the xenografted HaP-T1 induced tumor showed a more poorly differentiated and medullary form in its structure (**Fig. 8b**). Infiltrative cells, most of which were mononuclear cells, were observed to be surrounding the tumor more intensely than those of the homografted one (**Fig. 8b**).

Transmission electron microscopic findings of the homografted or xenograftrd HaP-T1 induced tumor are shown in **Figure 9a,b**. Morphological characteristics of tumor cells were rather similar both on syngeneic hamsters and athymic nude mice. Tubular structures consisted of cuboidal or columnar cells with abundant microvilli and some glycocalyces. These cells had a very high nucleocytoplasmic ratio and had round, oval or indented nuclei with prominent nucleoles. They had some mitochondria, free ribosomes, Golgi complexes and a few secretory granules. Tumor cells were attached to each other by apical junctional complexes and desmosomes (**Fig. 9a,b**). No basement membrane was shown. Many lipid droplets were observed in the cytoplasm of several tumor cells (**Fig. 9a**). There was much homogenious material, probably mucosubstance, in the lumen (**Fig. 9b**).

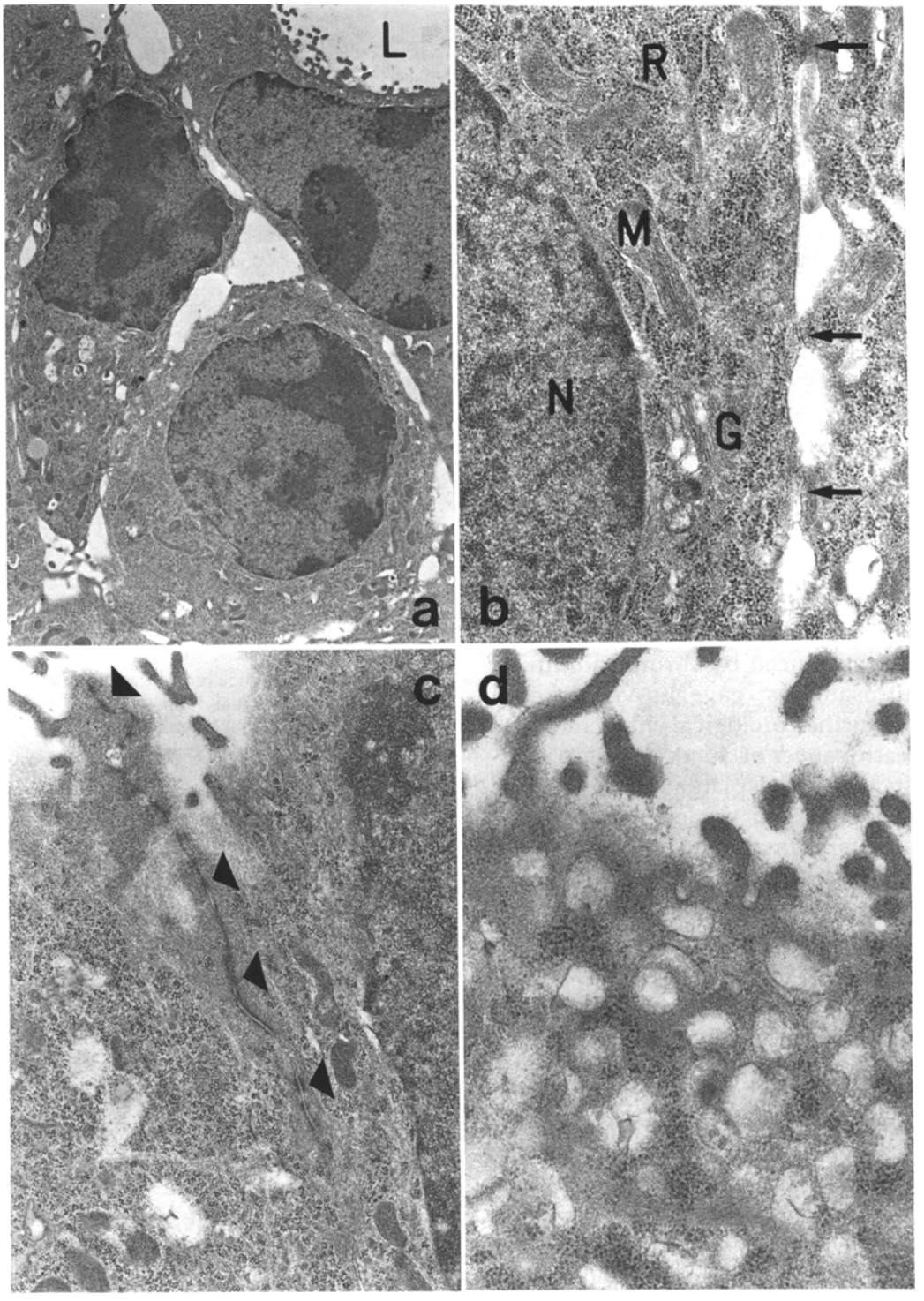

Fig 4 Electron micrographs of HaP-T1 cells in a monolayer culture.
a: A part of tumor cells, forming a luminar structure (L). ×4050
b: A higher magnification of a tumor cell indicating scarce mitochondria (M), Golgi complexes (G), rough endoplasmic reticulum (R) and abundant free ribosomes. Desmosomes are also found between the adjacent cells (arrows). ×22500
c: Junctional complexes (arrowheads) between adjacent cells facing a luminar structure. ×14400
d: Elongated and branched microvilli on a luminal survace and many secretory granule-like strctures in an apical cell cytoplasm. ×27900

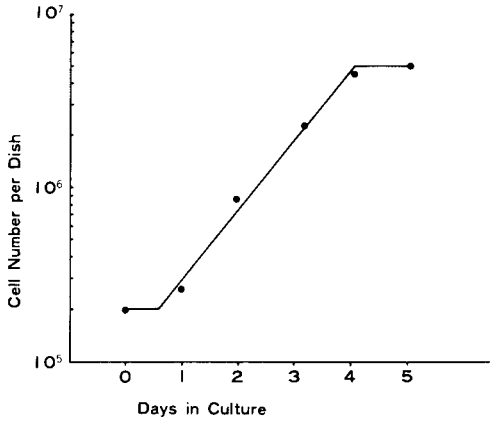

Fig. 5 Growth curve of HaP-T1 cells of the 40th passage *in vitro*.

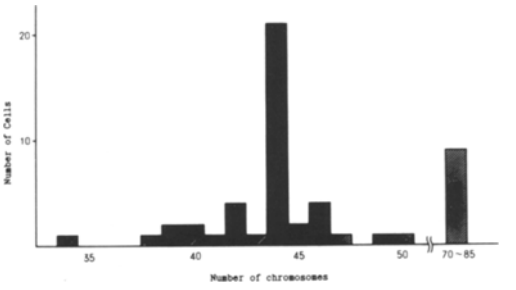

Fig. 6 Distribution of chromosomal numbers of HaP-T1 cells of the 40th passage.

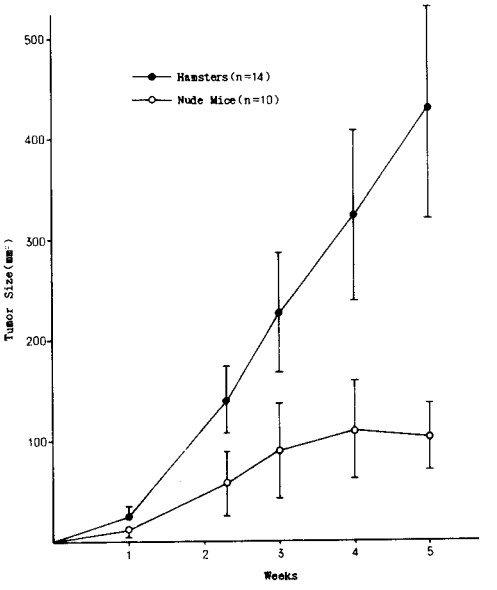

Fig. 7 Growth curves of HaP-T1 induced tumors *in vivo* when inoculated into syngeneic hamsters and athymic nude mice. mean±S.D.

Discussion

The study of pancreatic cancer has been hampered by the lack of a suitable animal model untill recent years. Development of the animal model of the pancreatic cancer in Syrian golden hamsters induced by chronic administration of nitrosamines[1] gave us very useful tools to clarify further biological characteristics of the pancreatic cancer of duct cell origin. The effort to establish transplantable pancreatic cancer lines from Syrian golden hamsters on both homologous animals and athymic nude mice have been attempeted by several authors[2-6]. However, there seemed to be some problems in transplantable models as pointed out by Townsend[7]. Because such lines required large numbers of animals to maintain passage, and tumor cell inoculum could not be quantified precisely in the experiment to investigate characteristics of cell growth or immunologic properties. Therefore establishment of continuous cell lines with high tumorigenicity in homologous animals is needed for the precisely quantitive experiment in the biological research of the pancreatic cancer *in vitro* and *in vivo*. In recent years, there have been a few reports describing nitrosamine-induced pancreatic cancer cell lines from Syrian golden hamsters including H2-T, WD-PaCa, CBP-TC and PDP-TC[7-9]. On the other hand, another type of transplantable pancreatic tumor lines derived from nafenopin or azaserine-induced acinal cell carcinoma in rats[10-14], spontaneous adenocarcinoma in rats[15] and methyl cholanthrene-induced adenocarcinoma in mice[16,17] have also been reported. Some of these transplantable tumors were developed into tissue culture[12-15,17]

In this report, we described the development of seven kinds of homologously transplantable tumor lines derived from BHP-induced pancreatic ductal adenocarcinomas arising in Syrian golden hamsters and the establishment of a cultured cell line designated HaP-T1.

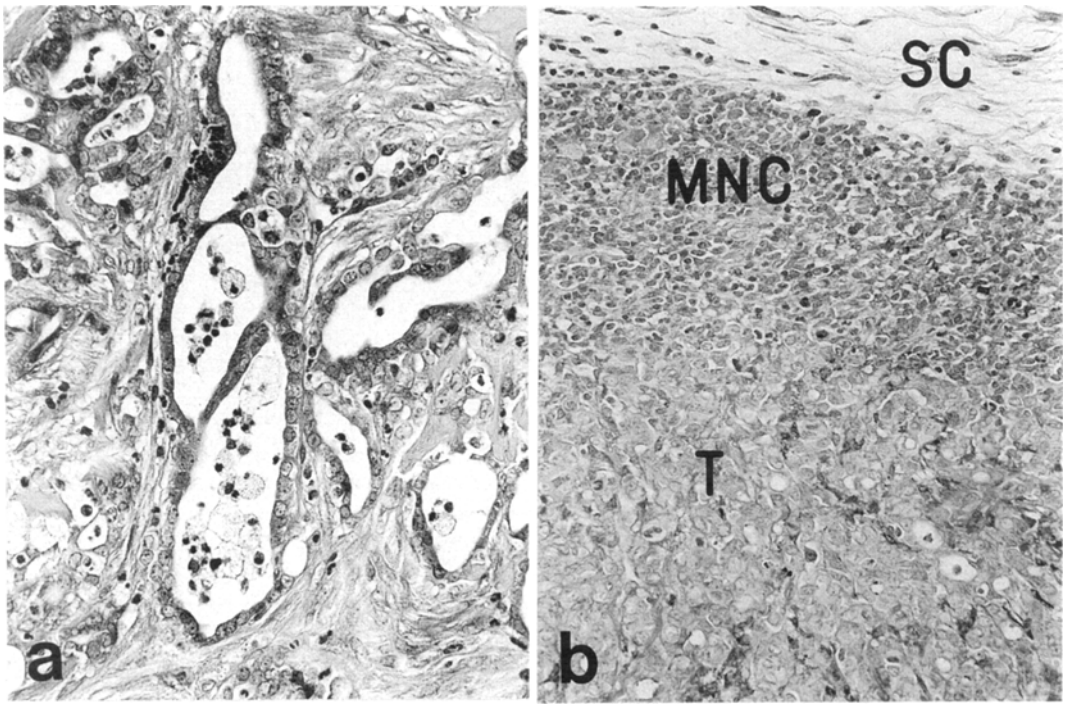

Fig. 8 Photomicrographs of a HaP-T1 induced tumor growing *in vivo*. H-E stain, ×200
a: Histological feature of a HaP-T1 induced tumor growing on a syngeneic hamster showing moderately differentiated adenocarcinoma.
b: Histological feature of a HaP-T1 induced tumor growing on an athymic nude mouse showing more poorly differentiated form in comparison with that on syngeneic animals. Dense infiltration of mononuclear cells (MNC) are found between the subcutaneous connective tissue (SC) and the tumor cells (T).

Our seven transplantable tumor lines showed very different growth characteristics respectively. No positive correlation between the growing velocity of each tumor line and its grade of histological differentiation was found. It seems to be very interesting that each of the seven transplantable pancreatic cancers themselves have apparently different intrinsic growth characteristics though they were induced with the same chemical agent in the syngeneic animals. Further experiments are needed to answer the question whether the variation in growing velocity among our seven transplantable lines is due to the difference of immunogenicity between each of the tumor lines or due to only the intrinsic variety of the growing characteristics of each tumor cell.

Very few reports have referred to the detailed characteristics of cultured cell lines derived from nitrosamine-induced pancreatic cancer in Syrian golden hamsters. Our results showed that the population doubling time of HaP-T1 *in vitro* was about 17 hours which was much faster than those of H2-T[7] and WD-PaCa[8] (28 and 36 hours, respectively). HaP-T1 seemed to show faster growth also in syngeneic animals than H2-T[7]. From these observations, HaP-T1 is a cell line which has a very fast growing velocity *in vivo* as well as *in vitro*. The HaP-T1 induced tumor showed a much faster growth when the cells were inoculated into the syngeneic hamsters than into the athymic nude mice. This result is completely opposite to that of Scarpelli[3]. we cannot explain the reason why Scarperi's line showed rather slower growth in the syngeneic hamsters, but his line might have a relatively strong immunogenicity which can be recognized by T cells of the syngeneic hamster.

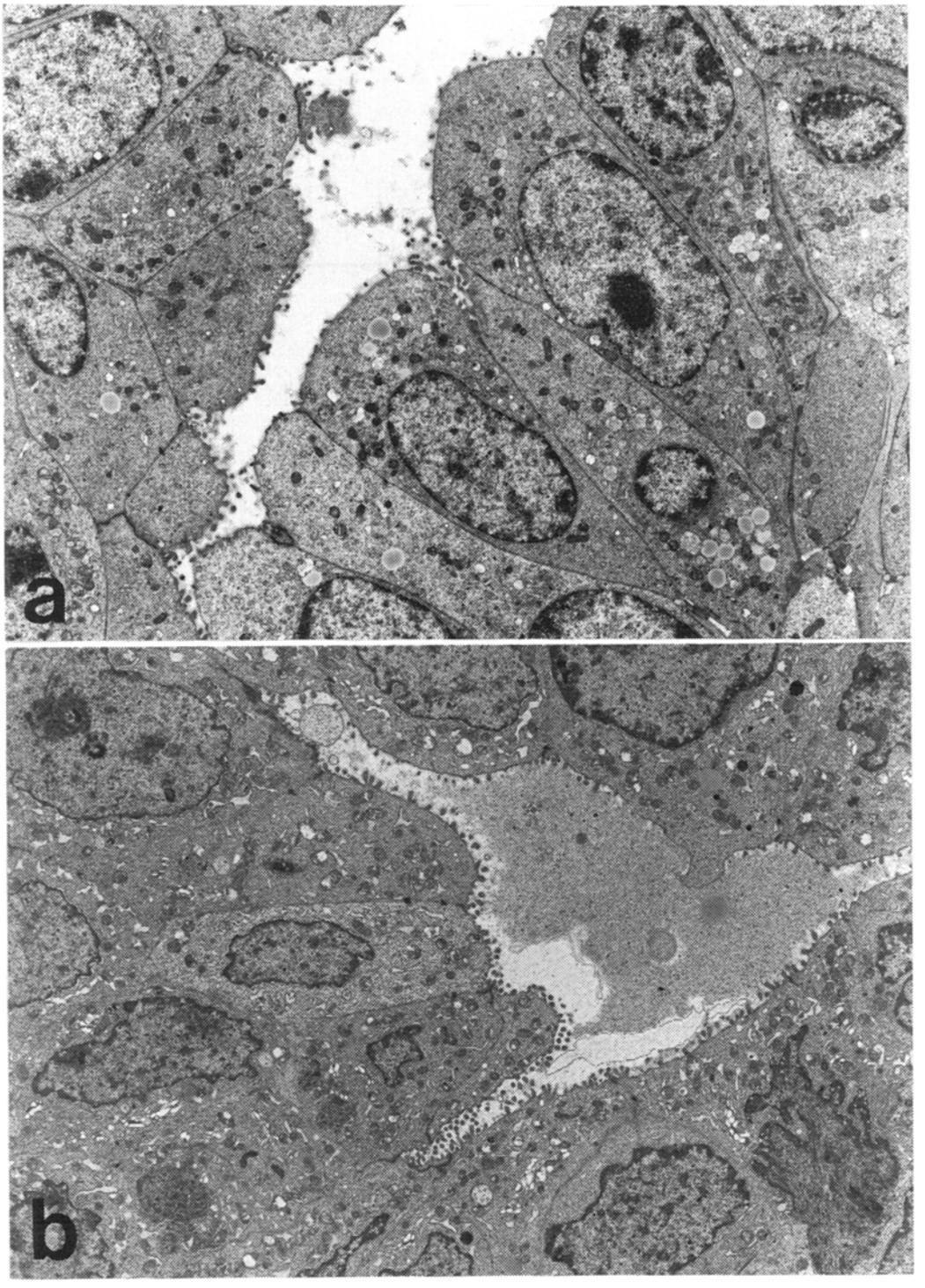

Fig. 9 Electron micrographs of a HaP-T1 induced tumor growing in vivo.
a: Fine structure of a HaP-T1 induced tumor growing on a syngeneic hamster, showing tubular structures consisted of columnar cells with abundant microvilli and scarce intracytoplasmic organelles. ×3780
b: Fine structure of a HaP-T1 induced tumor growing on an athymic nude mouse, showing multilayered tumor cells, forming a lumen, with many microvilli. Homogenuous materials, probably mucosubstance, are found in the lumen. ×3870

No investigation has referred to the chromosomal analysis of the cultured pancreatic cancer cell lines of the Syrian golden hamster. Scarpelli[3] reported that the distribution of chromosomal numbers of his tumor line in the nude mice indicated a hypodiploid with a modal number of 37 as compared to that of 44 in normal hamster somatic cells. The distribution of chromosomal numbers of HaP-T1 ranged from 34 to 85 with a modal number of 44. This result seems to be compatible with the fact that HaP-T1 is a cell line of Syrian golden hamster origin.

The histogenesis of the nitrosamine-induced pancreatic cancers in Syrian golden hamsters has been controversial. One of our coworkers studied the morphological aspects of our seven kinds of homologously transplanted pancreatic cancer lines with light and electronmicroscopy[18]. These pancreatic cancers were all adenocarcinomas and generally well or moderately differentiated, showing glandular patterns. The epithelial cells of the malignant glands often had secretory granules which seemed to contain glycoproteins and mucosubstances demonstrated with several techniques of electron microscopic histochemistry[18]. Therefore, we believe that our transplantable pancreatic cancer lines were derived from duct or ductular cells. Morphological examinations of HaP-T1 cells showed apparent epithelial characteristics even under *in vitro* conditions. The cells often formed glandular-like luminar structures and abundant microvilli, well formed desmosomes and many secretory granules were observed in these cells with electron microscopic examination. When HaP-T1 cells were inoculated into the syngeneic hamsters subcutaneously, the histological features of the induced tumor were very similar to those of the homologously transplanted pancreatic cancer line which was the original source of the cell culture. These results seem to supprot the view that HaP-T1 cells preserve their ability to form the morphological structures resembling pancreatic ducts or ductules. Of additional interest is the difference between the homografted HaP-T1 induced tumor and the xenografted one in both morphological and biological aspects. In our observation, histological examination of the xenografted HaP-T1 induced tumor revealed less amount of interstitial tissue among the tumor nests and more intense infiltration of inflammatory cells compared with that of the homografted HaP-T1 induced tumor. In addition, the xenografted HaP-T1 induced tumor also showed a more poorly differentiated structure than the homografted one. It has been well known that tumor cells should interact with surrounding interstitial tissue when tumor is growing and invading into adjacent tissues. In the case of xenograft, the tumor cells and the surrounding stroma should have different histocompatibility antigens and these differences may alter the fashion of interaction between the tumor cells and the stroma. These changes of the interaction may affect the kinetics of the tumor cells, the structural differentiation of the tumor tissue and the reaction of the interstitial tissue component to the tumor cells. Therefore, it should be emphasized that we have to consider these biological and morphological modifications when we use xenografted tumor models in oncological research.

We concluded that HaP-T1 is a well characterized and highly tumorigenic cultured cell line derived from pancreatic duct adenocarcinoma of Syrian golden hamsters and we believe that this cell line should be a new useful model contributing to the further investigation of pancreatic cancer.

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