

## Characterization of Two New Cancer Cell Lines Derived from Human Breast Tumors<sup>1</sup>

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### SUMMARY

Two human carcinoma lines, designated as HMC-1-8 and HMC-2-1, were established from metastatic pleural effusions of female patients with breast cancers. At the beginning of culture, these cells proliferated in floating fashion rather than adherent, and after culturing for one month, the cells were adapted to grow on the surface of plastic culture flasks. We studied several characteristics of the cells such as light and electronmicroscopic features, anchorage independent growth, tumorigenicity in nude mice and production of tumor antigens. Results obtained from these studies indicate that HMC-1-8 and HMC-2-1 are epithelioid cancer cells, and could serve as breast tumor cells for the biological analysis of this specific tumor.

**Key words:** Human breast cancer lines, HMC-1-8, HMC-2-1

### INTRODUCTION

The increasing incidence of patients with breast cancer is remarkable in recent years. In spite of a rather high survival rate of this cancer after the radical operation, it is usually known that once tumors recur in patients, they grow progressively, and patients succumb rapidly. The breast carcinoma is a somewhat particular tumor because the growth is partially dependent on sex hormones. However, and large part of the cell growth mechanism including hormone dependency is still unknown. Therefore, tumor cell lines derived from human breast cancers are valuable tools for research of this particular cancer. Since Lasfargues and Ozzello

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(1) reported in 1958, several breast cancer cell lines have been established(2, 3, 4, 5, 6, 7, 8, 9). However, because of their complex dependency on hormone for the cell growth, successful cultures have remained difficult, and in fact, success was only incidental.

In our previous study, it was indicated that HMC-1-8 and HMC-2-1 expressed the antigens that were recognized by autologous cytotoxic T cell clones and NK-like effector cells, respectively. In the present report, we demonstrate the establishment and characterization of two cell lines derived from metastatic pleural effusions of female patients with breast cancers.

## MATERIALS AND METHODS

### *Cell source and culture*

In this study, we attempted at first to establish two tumor lines, HMC-1 and HMC-2. HMC-1 was derived from a malignant pleural effusion in a 35 year old female patient who had undergone radical mastectomy 2 years before beginning the cell culture for infiltrating ductal carcinoma of the right breast. HMC-2 was obtained from a 60 year old female patient who had also undergone radical mastectomy 3 years prior to cultivation for infiltrating ductal carcinoma of the left breast. Several months before their death, they were diagnosed of local recurrences, and found to have metastatic pleural effusions. Thoracentesis was done frequently due to massive pleural effusions.

We have already reported the establishment of two cancer cell lines derived from pleural and peritoneal effusions of the pancreatic cancers, and in the present study, we utilized almost the same procedures for the cultivation of cells(10). Approximately 500 ml of metastatic pleural fluid was centrifuged at  $250 \times g$  for 10 min. The cell pellets were resuspended in 20 ml of RPMI-1640 supplemented with 10% fetal calf serum (FCS), 292  $\mu\text{g/ml}$  of L-glutamin, 100  $\mu\text{g/ml}$  of streptomycin and 100 units/ml of penicillin. The cells were carefully layered on a 20 ml of Ficoll-Conray density gradient (S. G. 1.082). After spinning down at  $1,000 \times g$  for 25 min at room temperature, the interface in which the tumor cells, macrophages, mesothelial cells, fibroblasts and lymphocytes were enriched was collected, and washed three times with RPMI-1640 medium. Then the cells were seeded in a 25 cm<sup>2</sup> culture flask (Falcon #3013, Oxnard, CA.), and cultured under 37 C in 5% CO<sub>2</sub> incubator. Approximately six hours later, the supernatants containing unattached cells were pooled, concentrated and seeded into other flasks. The supernatant of each flask was daily reseeded into other flasks for the first week, and in this manner, the newly seeded cells were enriched in such a way that tumor cells attached slowly.

In contrast, other more rapidly attaching cells, particularly macrophages, mesothelial cells and fibroblasts were selectively reduced. About one month after

the initiation of culture, the attaching cells such as non-malignant epithelioid cells were almost reduced, and floating cancer cells began to adhere onto the underface of culture flasks. Thereafter the cancer cells began to grow, and these were serially transferred into other flasks by treating with 0.5% trypsin (Sigma Chemicals Co., St. Louis, Mo.) plus 0.02% EDTA in a phosphate buffered saline (PBS) at weekly intervals. Both of HMC-1 and HMC-2 cells propagated in an adherent fashion as a cell monolayer rather than in floating style.

#### *Clone establishment*

At approximately 5 months of a successful culture, single cell cloning was performed for both cells, using 60 mm petri dishes (Falcon #3002) as described previously(11), and HMC-1-8 and HMC-2-1 clones were established. At this time these lines were assessed for mycoplasma contamination by using a mycoplasma stain kit (Flow Laboratories Inc., VA.) and both lines were found negative for mycoplasma contamination.

#### *Morphological examination*

A conventional Giemsa's staining of HMC-1-8 and HMC-2-1 clones was done at 38th and 24th passage generation, respectively, and light microscopical morphology of cells were observed. Simultaneously, electronmicroscopic features of the adherent cells in a tissue culture chamber (Lab-Tek #4880, Miles Co., IL.) and tumor tissue growing in nude mice were observed. The cells were fixed with 0.1 M cacodylate buffer and washed with 7.5% sucrose for 5 min. The fixed cells were dehydrated in a graded series of ethanol from 50-100% and propylene oxide, and were embedded in epon-propylene oxide. The thin sections were stained with 5% uranyl acetate and observed.

#### *Doubling time of cells*

$5 \times 10^4$  HMC-1-8 and HMC-2-1 at 27th and 24th passage generations, respectively, were seeded into 25 cm<sup>2</sup> culture flasks. After complete trypsinization of the cell monolayer, the free cells were counted at 6 hr interval for five days, and the doubling time of cells were determined as described previously(11).

#### *Tumorigenicity in soft agar*

The cells were cultured in soft agar as reported previously(11). Briefly,  $10^2$  or  $10^3$  of HMC-1-8, HMC-2-1 and BALB/c mouse colon line C-C36 were plated in dishes (Falcon #3002) containing 0.3% agar, and incubated under 37 C at 5% CO<sub>2</sub> incubator. The number of colonies in this agar were scored with % plating efficiency after 2 and 3 weeks of cultivation. C-C36 line(12) was used for a positive

anchorage-independent cell growth in this agar of the experiment. This cell line has been shown previously to be highly tumorigenic in nature *in vitro* as well as *in vivo*.

#### *Tumorigenicity in nude mice*

HMC-1-8 and HMC-2-1 cells growing *in vitro* were harvested by trypsinization, and  $10^6$  cells per mouse were injected subcutaneously in the back of five BALA/c nude mice obtained from CLEA Japan Co., Shizuoka, Japan. The mice were observed weekly for the tumor growth. Light and electronmicroscopic features of some of tumors that developed in the mice were removed, and were observed microscopically.

#### *Assays for tumor-associated antigens*

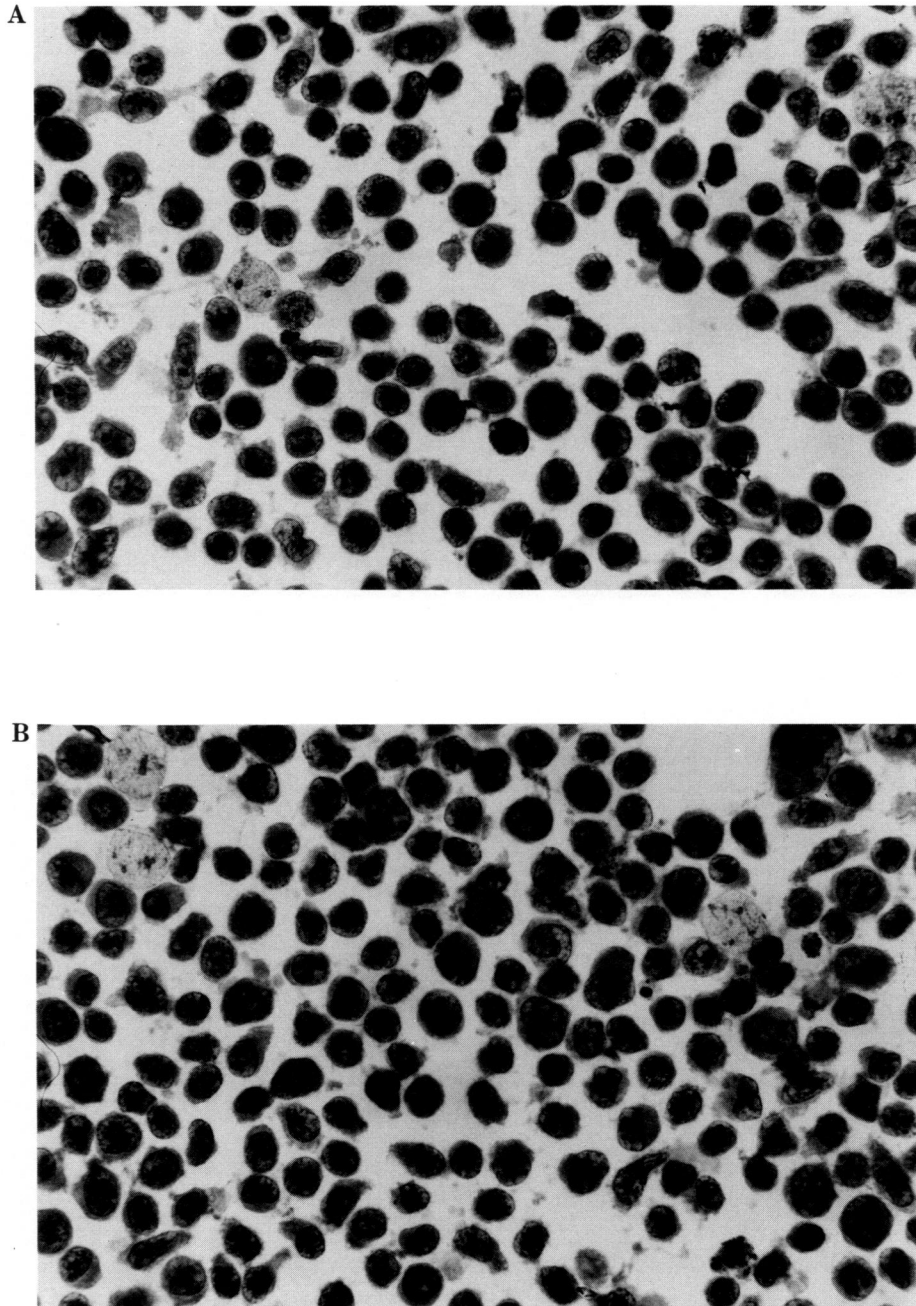
$10^5$  cells were seeded into tissue culture flasks. After culturing for 72 hr, the cells were multiplied to approximately  $10^6$  cells. These supernatants were assessed for the presence of the tumor-associated antigens such as  $\alpha$ -fetoprotein (AFP), CA19-9, carcinoembryonic antigen (CEA), ferritin and tissue polypeptide antigen (TPA) using radioimmunoassays by Ohtsuka Assay Laboratories, Tokushima, Japan. For the control, cell free RPMI-1640 medium supplemented with 10% FCS and normal human serum were employed for the measurement of these antigens.

## RESULTS

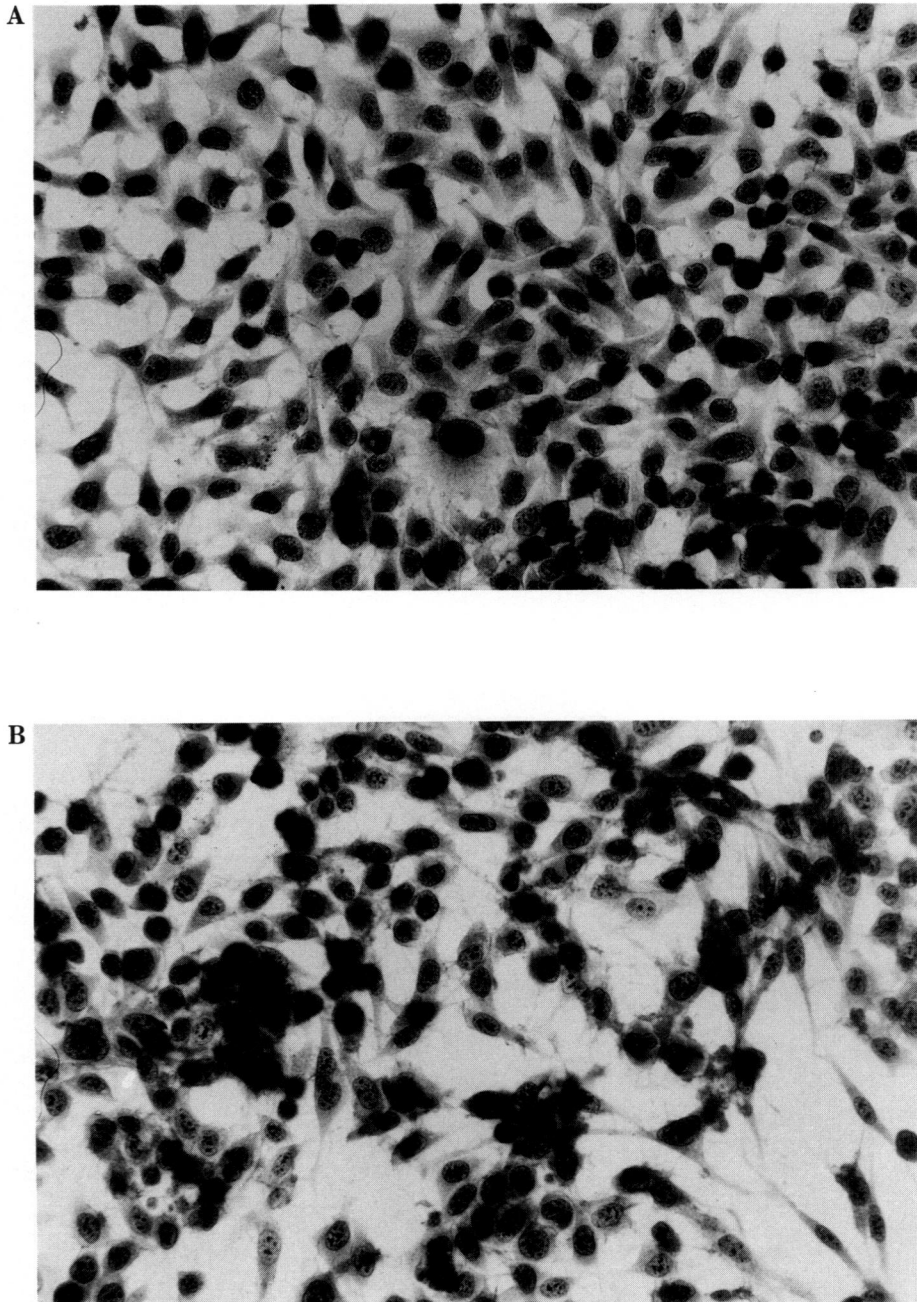
#### *Morphological characteristics*

As shown in Fig. 1, most of tumor cells in the pleural fluid from two breast cancer patients showed round or oval shapes of various sizes with a single or divided nucleus. At primary culture, the tumor cells began to grow *in vitro* in RPMI-1640 medium supplemented with 10% FCS in a floating fashion. After culturing for one month, both cases indicated that adherent tumor cells began to proliferate on the bottom of the plastic culture flasks and showed a monolayer of the cells with partial piling-up foci (Fig. 2). After a single cell cloning, these two cultured cells were designated as HMC-1-8 and HMC-2-1. The histology of these tumors developed in nude mice showed undifferentiated or anaplastic carcinomas (Fig. 3).

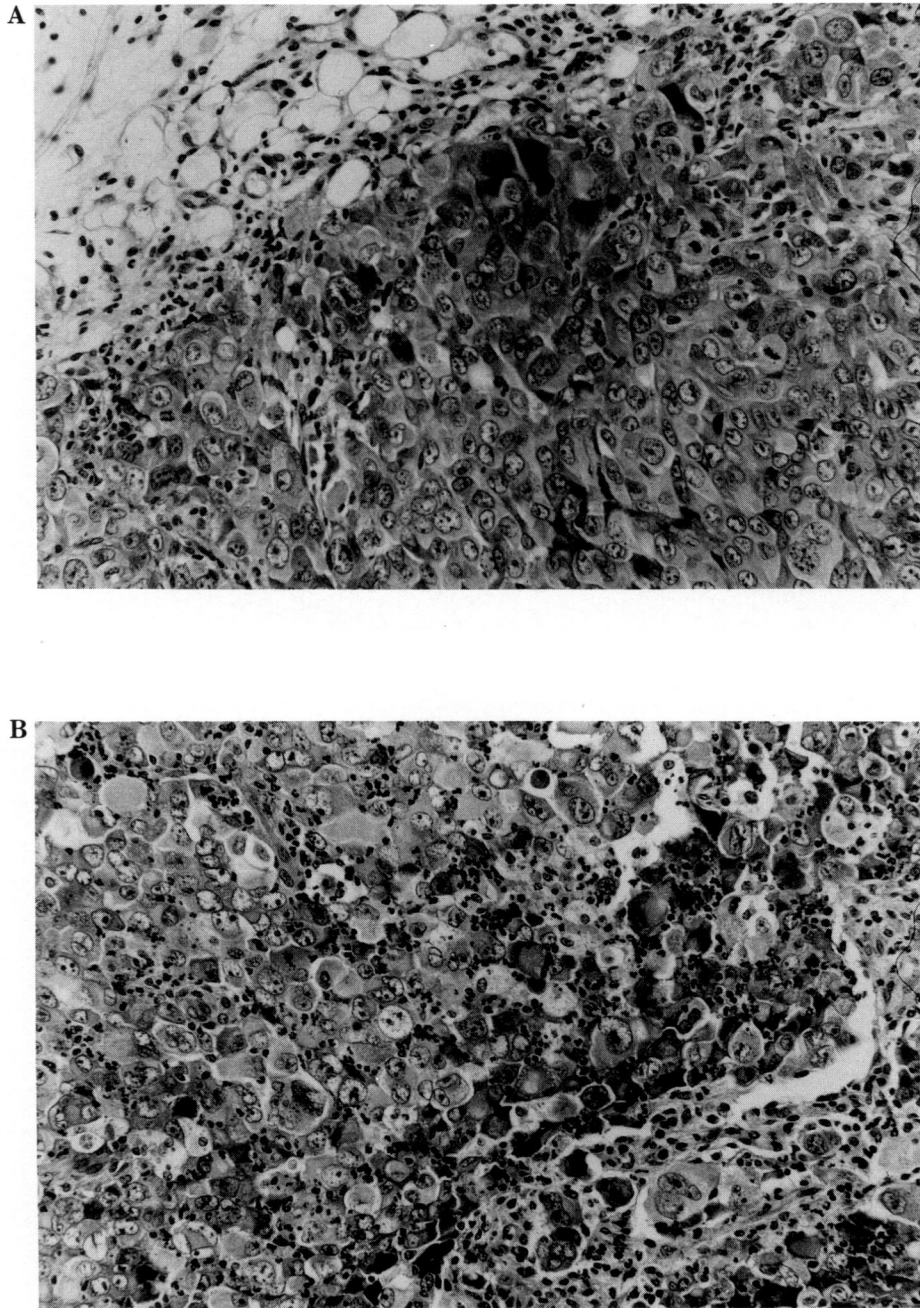
We also examined the ultrastructures of cells growing *in vitro* and tumors that developed in nude mice. Fig. 4 demonstrates that HMC-1-8 and HMC-2-1 have a round-shaped cytoplasm and large nucleus with clear nucleoli. Both tumors developed in nude mice showed desmosome-like intercellular junctions and a few microvilli on the cell surface. A few of microorganellas such as mitochondria, rough endoplasmic reticulum and secretory granules were also observed. However, virus-like particles and mycoplasmas were not detected (Fig. 5). These data



**Fig. 1** A microscopic view of uncloned tumor cells (A, HMC-1; B, HMC-2) in the primary culture. Most of cells were growing in floating fashion. Giemsa stain,  $\times 400$ .

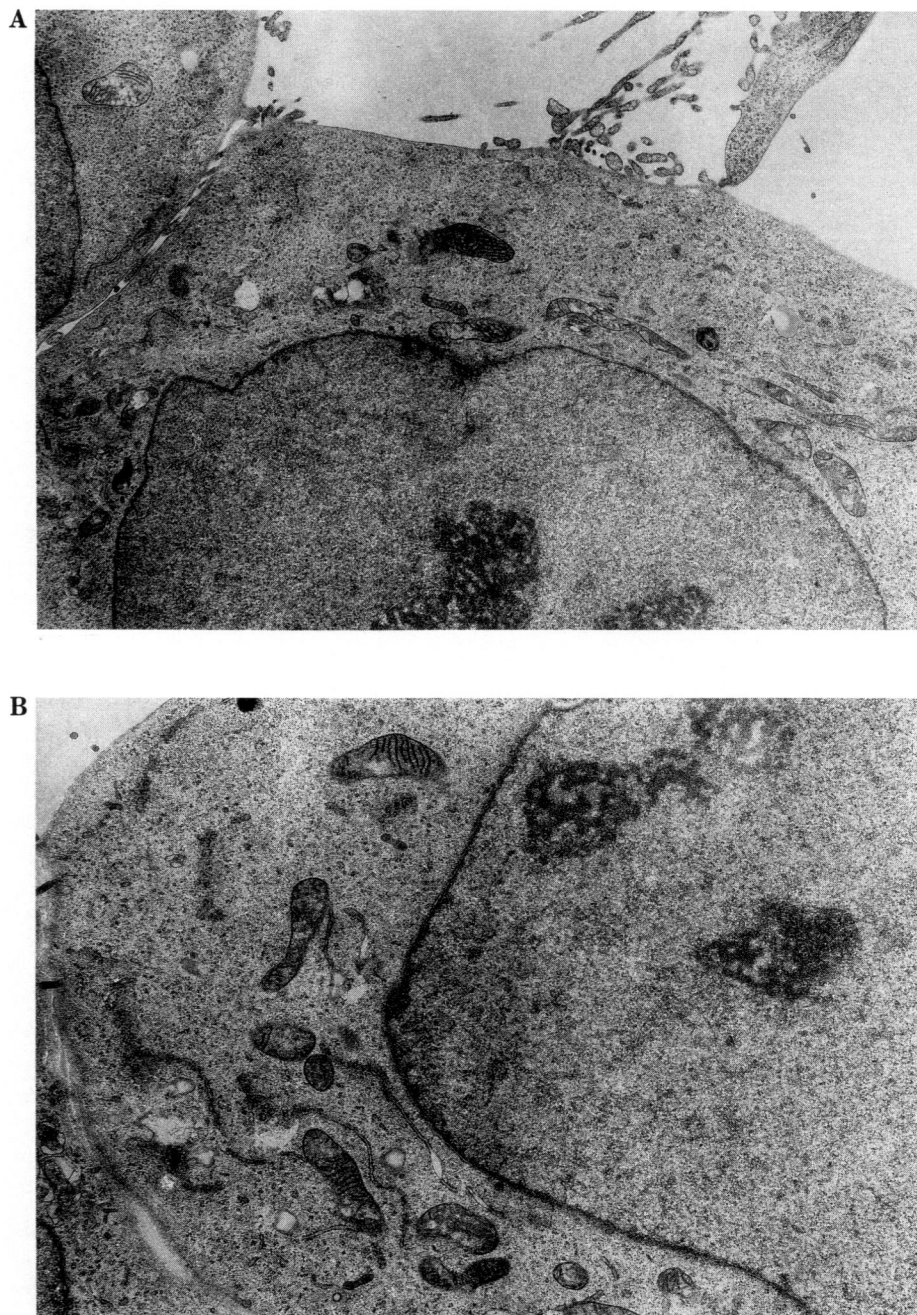


**Fig. 2** A microscopic view of continuous growing cells in vitro. Both of HMC-1-8 (A) and HMC-2-1 (B) showed cell growth in adherent fashion rather than floating. Giemsa stain,  $\times 400$ .



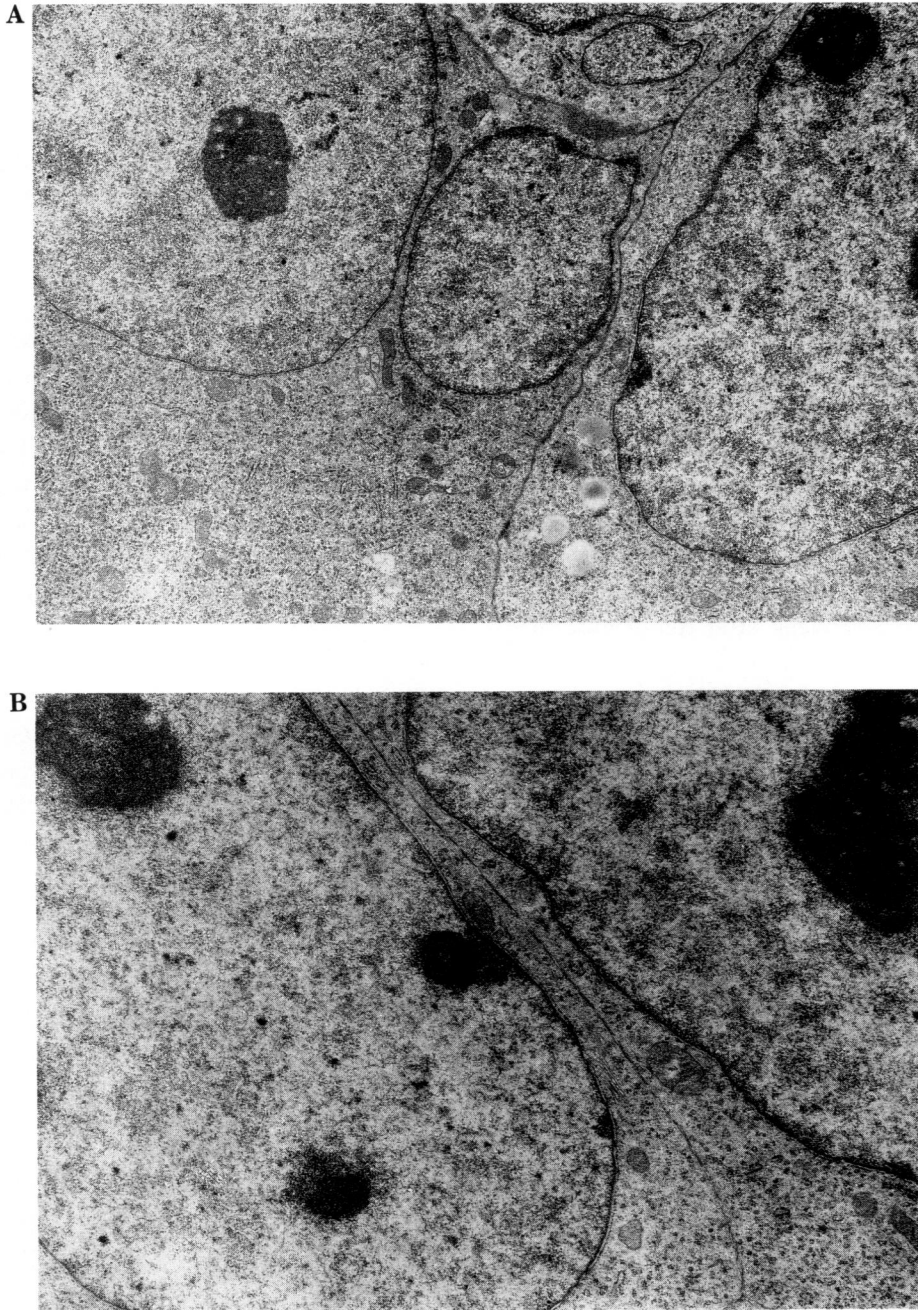
**Fig. 3** Histology of tumors developing in nude mice (A, HMC-1-8; B, HMC-2-1), indicating undifferentiated or anaplastic carcinomas. H. E.,  $\times 200$ .





**Fig. 4** Electronmicroscopic views of HMC-1-8 culturing in vitro (A,  $\times 3,300$ ) and HMC-2-1 (B,  $\times 5,000$ ).





**Fig. 5** Electronmicroscopic views of HMC-1-8 (A,  $\times 8,300$ ) and HMC-2-1 (B,  $\times 6,600$ ) tumors deloping in nude mice.

strongly suggested that both of HMC-1-8 and HMC-2-1 cells were epithelioid in nature.

#### *Growth characteristics*

The doubling time was determined during the exponential phase of cell growth. It was shown that HMC-1-8 and HMC-2-1 had 26 and 22 hr of doubling time, respectively.

#### *Tumorigenicity of cells*

In order to analyze the growth capability of these cells, anchorage-independent growth in soft agar was tested. It was shown in Table 1 that both cells could form colonies although the plating efficiency of these cells was relatively low. Under these experimental conditions, C-C36 showed a high plating efficiency. For the assessment of tumorigenicity *in vivo*  $10^6$  tumor cells were injected subcutaneously into five nude mice. The data showed that both of these cells were tumorigenic in nude mice. Actually one or two out of each five mice injected with HMC-1-8 or HMC-2-1, respectively, demonstrated tumor development. All tumors were found until 12th week after tumor injections.

#### *Tumor-associated antigens*

$10^5$  of HMC-1-8 and HMC-2-1 cells were cultured for 3 days in 5 ml RPMI-1640 medium supplemented with 10% FCS per flask, and the culture supernatants were assayed by radioimmunoassay for the presence of tumor-associated antigens. As

**Table 1** *Anchorage-independent growth in 0.3% agar and tumorigenicity in nude mice of HMC-1-8 and HMC-2-1 clones.*

cells	inoculum		% plating efficiency <sup>a)</sup> at		tumor growth <sup>b)</sup> in nude mice
	0.3% agar	nude mice	2 weeks	3 weeks	
HMC-1-8	$10^2$		$20.4 \pm 0.4^c)$	$27.7 \pm 5.5$	
	$10^3$		$19.7 \pm 3.8$	$24.1 \pm 5.3$	
		$10^6$			2/5
HMC-2-1	$10^2$		$7.2 \pm 1.5$	$17.0 \pm 4.3$	
	$10^3$		$13.2 \pm 3.0$	$17.2 \pm 2.4$	
		$10^6$			1/5
C-C36	$10^2$	N. D.	$87.0 \pm 6.2$		
	$10^3$		N. D.	$55.6 \pm 8.0$	

<sup>a)</sup> % plating efficiency in 0.3% agar was calculated: (No. of clusters-No. of original cell aggregates)  $\times$  100/No. of viable cells plated.

<sup>b)</sup> The data was expressed as No. of mice with tumor/No. of mice injected. The tumor development was observed until 12 weeks after cell inoculation.

<sup>c)</sup> Mean  $\pm$  S. D.

**Table 2** *Production of tumor-associated antigens in the culture supernatants of HMC-1-8 and HMC-2-1 clones.*

cells	AFP	CEA	Ferritin	CA19-9	TPA
HMC-1-8 <sup>a)</sup>	5.0<	1.0<	23.2	5.0<	1093.1
HMC-2-1	5.0<	1.0<	9.1	5.0<	639.1
medium control <sup>b)</sup>	5.0<	1.0<	5.0<	5.0<	30.0<

<sup>a)</sup> 10<sup>5</sup> cells were seeded into tissue culture flasks. After culturing 72 hr, the culture supernatants were harvested, and were assessed for the presence of tumor-associated antigens.

<sup>b)</sup> RPMI-1640 plus 10% fetal calf serum.

shown in Table 2, TPA in these supernatants showed remarkably high values, that is, 1093.1 for HMC-1-8 and 639.1 IU/L for HMC-2-1. The supernatant of HMC-2-1 culture also showed an increased level of ferritin. However, cell free medium control containing 10% FCS indicated that it was an undetectable level for TPA. Other tumor-associated antigens such as CA19-9, AFP and CEA were not increased, and were actually below detectable values.

#### DISCUSSION

It is generally considered that the establishment of tumor cell lines is usually incidental, and successful culture depends upon the materials from the patients. However, as some investigators have reported previously(7, 13, 14), the culture of cells was more successful when using metastatic pleural or peritoneal effusions rather than primary solid tumors. This may indicate that metastatic cells growing in floating fashion in the body cavity are already of the adapted form of cells in the milieu that are different from the primary tissues, and therefore, the tumor cells in these metastatic fluid showed an enhanced form for cell establishment. We have succeeded previously in establishing cell lines derived from metastatic pleural effusions of pancreatic cancers(10).

There is no question that these effusions have several advantages for culturing ; we can obtain many free tumor cells with high viability and no damage by protease digestion such as trypsin, collagenase and many other digestive agents. Furthermore, it seemed that the most important point for these floating cells obtained from effusions might be the existence of their favorable circumstances such as the metastatic body cavity in which tumor cells were proliferating.

The cytological morphology of each of tumor cell line closely resembled each other in that they had characteristics seen in aspiration biopsies. These cultured cells are clearly epithelial in nature in vitro as well as in vivo, and they exhibited ultrastructural features that are characteristic of the epithelioid cells such as

desmosome-like intercellular junctions. Moreover, some glycogen granules were observed in the cytoplasmic portion.

These two lines also showed their malignant potentials as suggested by anchorage-independent growth *in vitro*. This potential of cells in soft agar is a most reliable parameter to indicate the neoplastic nature of cells(12, 13). Indeed, as Drebin *et al*(16) reported previously, NIH3T3 cells transformed by various oncogenes developed macroscopic colony formations in soft agar, but non-transformed NIH3T3 cells did not grow anchorage-independently. As compared to high tumorigenic murine colon tumor line C-C36, these cells showed a rather low plating efficiency in soft agar. However, these lines developed tumors in nude mice. Therefore, it is considered that both of HMC-1-8 and HMC-2-1 have neoplastic potentials *in vitro* and *in vivo*.

Our previous study showed that these cells were susceptible to the cytotoxicity of host killer cells. It suggests that HMC-1-8 expressed the antigen that was recognized by autologous cytotoxic killer T cell clones, and that HMC-2-1 was destroyed by NK-like effector cells of autologous origin. These phenomena are very interesting when considered as the host immune surveillance mechanisms against malignant tumors, since the data indicated that HMC-1-8 and HMC-2-1 expressed different tumor antigens that were target structures by the host effector cells. Furthermore, although there were no differences in *in vitro* growth characteristics among HMC-1 clones such as HMC-1-8 and HMC-1-7 (data not shown), HMC-1-8 clone was shown to express antigens that were involved in the cytotoxic mechanism by autologous T<sub>HMC-1</sub> killer cells. This heterogeneity of antigen expression among HMC-1 clones is another subject of our research interests.

These two cancer tissues obtained by radical mastectomy indicated that the tumors were positive for 17  $\beta$ -estradiol and progesterone receptors. In contrast, these established forms *in vitro* showed a disappearance of these receptors (data not shown). With respect to the hormone receptors of the breast cancers, the past several years have seen remarkable progress toward the understanding of cell growth mechanisms depending upon sex hormones(17, 18, 19, 20, 21). For the patients with breast cancer, chemoendocrine treatment as routine therapy has been taken. Some lines from the breast cancers such as MCF-7 still retain receptors for sex hormones(7, 22, 23). We do not know why these receptors of HMC-1-8 and HMC-2-1 disappeared from the cells. However, it is clear that the present cell lines might provide the validity for *in vitro* assays of human breast cancers in studying the malignant potential of cells.

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