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CARCINOEMBRYONIC ANTIGEN PRODUCTION IN HUMAN GASTRIC CANCER CELL LINES *IN VITRO* AND IN NUDE MICE

Teiichi MOTOYAMA and Hidenobu WATANABE
First Department of Pathology, Niigata University School of Medicine, Asahimachi-dori 1-757, Niigata 951

A quantitative study of carcinoembryonic antigen (CEA) was made in nine human gastric cancer cell lines. Six of them were found to produce CEA *in vitro*. The production of CEA in the three cell lines derived from well differentiated tubular adenocarcinomas began at the mid-exponential phase of cell growth and reached its peak at the late stationary phase, the amount of CEA per 10^5 cells and the frequency of CEA-positive cells on immunostaining increased with culture time. In contrast, CEA in the three cell lines derived from poorly differentiated adenocarcinomas including a signet-ring cell carcinoma was produced immediately after plating and the amount of the antigen per 10^5 cells and the frequency of CEA-positive cells were almost constant throughout the cell growth phases. Serum CEA content in nude mice was low or not detectable in the case of subcutaneous heterotransplantation of gastric cancer cells, irrespective of CEA productivity of the cell lines *in vitro*. Intraperitoneal inoculation, however, led to high CEA levels in sera of nude mice bearing human gastric cancers. No significant difference was found between the two kinds of inoculation in terms of the total tumor weight and the frequency of CEA-positive cells in tumor tissues. One reason for the above findings may be that the transport of CEA in the subcutaneous tumors to the systemic blood flow is hindered.

Key words: Carcinoembryonic antigen — Gastric cancer cell line — Transplanted tumor

Carcinoembryonic antigen (CEA) was first identified in the human fetal gut and in colon carcinomas by Gold and Freedman[7] in 1965, but it was subsequently shown to be associated with a range of normal and malignant tissues,[24] including nongastrointestinal cancers.[8] Although the lack of site-specificity has detracted from the utility of CEA as a tumor marker for screening purposes, CEA remains an important adjunct to assist with the management of patients known to have CEA-producing tumors. In these cases, quantitative serial CEA values may be useful in monitoring the success of surgery or chemotherapy and in the earlier detection of recurrences.[1, 14] High levels of CEA may also be useful as an indicator of the primary site in metastatic disease of unknown origin.[11]

Some CEA-producing cell lines have been established in culture,[4, 5, 6, 23] but they have not been fully investigated as regards the manner of CEA production or the particular properties of the CEA-producing cells in the cancers. In the present paper we describe the characteristics of the CEA-producing cells in human gastric cancer cell lines *in vitro* and in nude mice.

MATERIALS AND METHODS

Cell Lines The nine cell lines used throughout this study were derived from various types of human gastric cancers. Five cell lines MKN1, MKN7, MKN28, MKN45 and MKN74 were established in our laboratory.[9, 18] Cell lines

KATO-III[21] OKAJIMA[18] and MK2[18] were kindly donated by Dr. M. Sekiguchi (Institute of Medical Science, University of Tokyo, Tokyo), Dr. M. Shimoyama (National Cancer Center Hospital, Tokyo) and Dr. J. Inoue (Tokyo Medical University, Tokyo), respectively. The cell line SCH, established by Oboshi *et al.*,[19] originated from stomach choriocarcinoma of a male patient. The cloned cell lines MKN28cl-4 and MKN45cl-2 were derived from MKN28 and MKN45, respectively.

Cell Cultivation for Determination of Growth Kinetics After three subculturings at the mid-exponential phase, 2×10^5 viable cells were plated in a 60 mm plastic culture dish in 3 ml of RPMI-1640 medium (Nissui Chemicals, Tokyo) supplemented with 10% fetal bovine serum and 200 μg/ml of kanamycin sulfate. The cultures were incubated at 37° in an atmosphere of 5% CO_2 in air at 100% humidity.

Twenty-four hours after the transfer and every 48 hr thereafter, cells in 2 dishes were harvested for calculation of the cell number until the 17th day of subcultivation. Culture media were collected and centrifuged at 3000 rpm for 5 min, and the supernatant was stored at −70° for CEA assay.

Cell Cultivation for Cytochemical Studies After three subculturings at the mid-exponential phase, approximately 1×10^4 cells were inoculated into a Lab-Tek chamber slide (22×22 mm in size) (Miles, Naperville, Ill.). Twenty-four hours after the transfer and every 48 hr thereafter, the media were refreshed. The cells on the slide were fixed with Bouin's solution for immunocytochemistry, on the 5, 9 and 13th days after inoculation. For mucus stains, the cells were fixed with a solution of equal parts of ethyl ether and 95% ethyl alcohol. McManus' method for neutral glycoprotein (periodic acid-Schiff, PAS) and the alcian blue method for acidic mucosubstances at pH 2.5 and pH 1.0 were applied.

Animals Males and female athymic nude mice (*nu/nu*) with a BALB/c genetic background (CLEA Japan, Tokyo) were used. At the start of experiments the mice were 5 to 7 weeks old and they were kept under specific pathogen-free conditions in vinyl film isolaters.

Heterotransplantation Approximately 1×10^7 cells were inoculated into either the subcutaneous tissue of the back or the peritoneal cavity. The size of transplants was measured (three diameters) once or twice a week. The animals were killed when the tumor became 2 cm or larger in the greatest diameter or 5 to 6 weeks after the inoculation. Sera of tumor-bearing mice and tumor tissues were obtained at sacrifice. The tissue extracts were obtained through perchloric acid treatment. Samples were stored at −70° before analysis. For immunohistochemistry, the specimens were fixed with Bouin's solution.

Assay of CEA CEA levels were measured by radioimmunoassay with the double-antibody method (CEA-IRE-SORIN, Paris).

Immunocytochemical Detection of CEA For immunocytochemical detection of CEA, cells or tissues fixed with Bouin's solution were used. The specimens were stained by the indirect immunofluorescence method. The horse anti-CEA sera were kindly supplied by Prof. H. Hirai (Hokkaido University School of Medicine, Sapporo). The sera were absorbed with human spleen extract in order to avoid staining of non-specific cross-reacting antigen (NCA).[17,25] Preliminary examinations showed that colo-rectal cancers, gastric cancers and fetal colonic mucosa exhibited strong reactions with the sera. No human tissue reacted with the sera absorbed with stomach or colon cancer tissues with high CEA contents.

Results

CEA Productivity in Gastric Cancer Cell Lines CEA was detected in culture media of 6 out of 9 cell lines (Table I). All 3 cell lines derived from well differentiated tubular adenocarcinomas and 3 out of 4 cell lines derived from poorly differentiated adenocarcinomas, including one signet-ring cell carcinoma, were producers of CEA *in vitro*. CEA levels per 10^5 cells were far higher in the latter 3 cell lines than in the former 3 cell lines. The latter 3 cell lines were also consistently positive for mucus stains (Table I). The cloned cell lines MKN28cl-4 and MKN45cl-2 showed CEA productivities similar to those of the parental cell lines (Table I).

Kinetics of CEA Production The total CEA yield increased with culture time in all of the CEA-producing cell lines. In the cell lines MKN28, MKN7 and MKN74, CEA was not detectable at the early exponential phase and the total yield and the amount of the antigen per 10^5 cells increased gradually with culture time as shown in Fig. 1A. In the cell line MKN45, the CEA was detectable even at the early exponential

Table I. CEA Content in Media and Mucus Stains of Cultured Gastric Cancer Cells

Cell line	Histological type of origin	Medium CEA[a]		Mucus stain[b]		
		ng/ml/48 hr	ng/10^5 cells/48 hr	PAS	Alcian blue pH 2.5	Alcian blue pH 1.0
MKN7	Well differentiated tubular adenocarcinoma	7	1	−	−	−
MKN28	Well differentiated tubular adenocarcinoma	160	6	−	−	−
MKN74	Well differentiated tubular adenocarcinoma	17	1	−	−	−
MK2	Poorly differentiated adenocarcinoma	nd[c]	nc[d]	±	−	−
MKN45	Poorly differentiated adenocarcinoma	1973	37	++	+	−
OKAJIMA	Poorly differentiated adenocarcinoma	3025	168	+++	+++	±
KATO-III	Signet-ring cell carcinoma	1384	62	+++	++	+
MKN1	Adenosquamous carcinoma	nd	nc	−	−	−
SCH	Choriocarcinoma	nd	nc	−	−	−
MKN28cl-4	Cloned MKN28	102	4	−	−	−
MKN45cl-2	Cloned MKN45	1894	39	+	+	−

a) The CEA levels were assayed on the 13th day.
b) −, negative; +, weakly positive, ++, moderately positive; +++, strongly positive.
c) nd: not detectable (<5 ng/ml).
d) nc: not calculable.

phase and the total yield of the antigen increased in parallel with the cell growth curve; the CEA amount per 10^5 cells was slightly elevated at the stationary phase of cell growth (Fig. 1B). The CEA amounts per 10^5 cells of KATO-III cell line and OKAJIMA cell line were not increased at all in spite of the marked increase in the total yield of CEA with culture time (Fig. 1C and 1D).

Immunocytochemical Staining of the Cell Lines with Anti-CEA Serum In the cell lines MKN28, MKN28cl-4 and MKN74, the frequency of positively stained cells with the anti-CEA sera was zero at the 5th culture day and was very low even at the 13th culture day. On the other hand, the frequency of CEA-positive cells was very high in the cell lines MKN45, MKN-45cl-2, KATO-III and OKAJIMA but was almost constant throughout the growth phases (Table II). Positively stained cells in the cell lines MKN28, MKN28cl-4 and MKN74 were relatively weakly fluorescent (Fig. 2), but the cells in the MKN45, MKN45cl-2, KATO-III and OKAJIMA cell lines were strongly fluorescent (Fig. 3).

CEA Content in Sera and in Tumors of Nude Mice Bearing Human Gastric Cancer Serum CEA levels were also lower in mice transplanted either subcutaneously or intraperitoneally with well differentiated tubular adenocarcinoma than in mice with poorly differentiated adenocarcinoma, paralleling the situation in the culture media. Serum CEA levels were low in the cases of subcutaneous heterotransplantation irrespective of the cell lines involved. On the other hand, intraperitoneal inoculation of the cancer cells resulted in higher levels of serum CEA in 2 cell lines examined. No significant difference of CEA content in the tumor tissues was found between subcutaneous and intraperitoneal hetero-transplantation of cloned cell lines, MKN-28cl-4 and MKN45cl-2. Total tumor weights

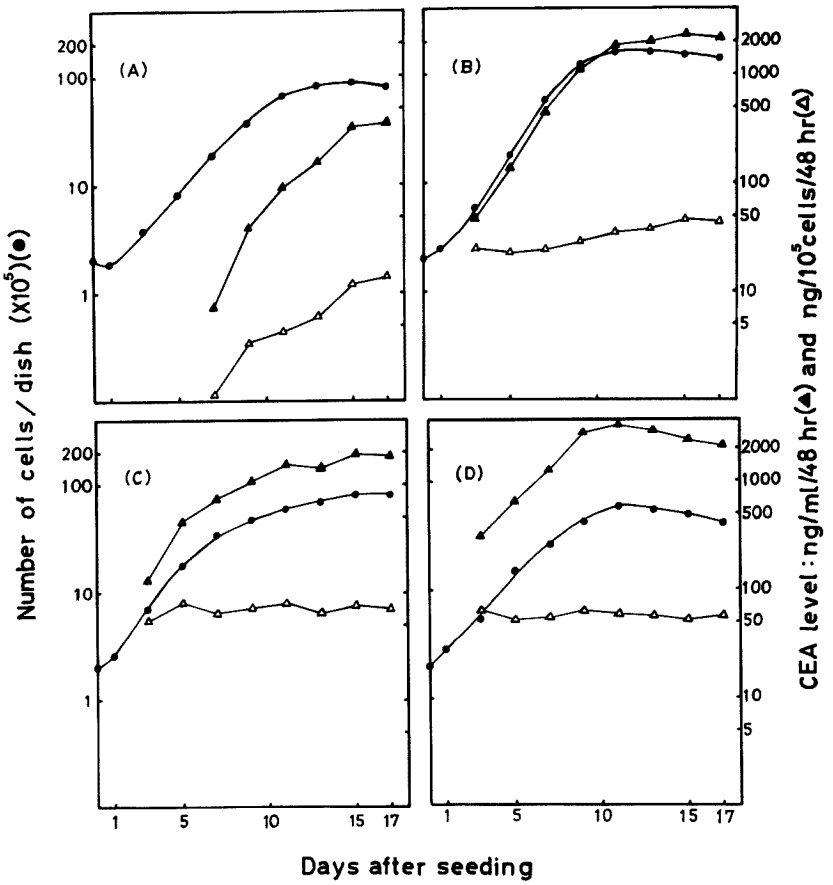

Fig. 1. Growth curves and CEA levels in media

The CEA production in MKN28 cells (A) was relatively low and dependent on the growth phase of the cells. On the other hand, the CEA production in MKN45 cells (B), KATO-III cells (C) and OKAJIMA cells (D) was high and was only slightly or was not dependent on the growth phase of the cells.

Table II. Frequency of CEA-positive Cells in Gastric Cancer Cell Lines *in vitro*

Cell line	Positive cells[a] (%)		
	5th day	9th day	13th day
MKN7	nt[b]	nt	nt
MKN28	0	0.8±0.2	4.9±0.4
MKN74	0	0	0.6±0.3
MK2	0	0	0
MKN45	96.4±1.1	96.5±1.3	96.4±1.3
KATO-III	85.7±3.6	85.0±2.7	87.4±4.3
OKAJIMA	99.3±0.6	99.2±0.8	99.3±0.6
MKN1	0	0	0
SCH	0	0	0
MKN28cl-4	0	0.7±0.1	3.7±0.8
MKN45cl-2	98.5±0.7	98.4±0.4	98.9±0.3

a) Mean±SD (n=5).
b) nt: not tested.

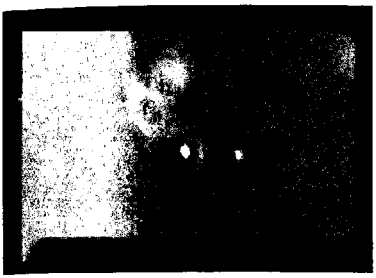

Fig. 2. Immunofluorescence micrography of MKN28cl-4 cells in a 13-day culture. ×200

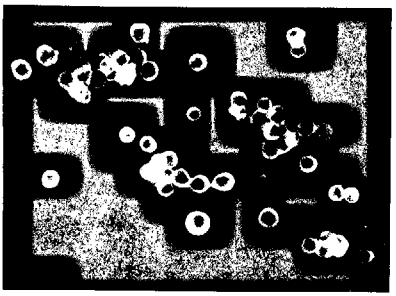

Fig. 3. Immunofluorescence study on MKN45cl-2 cells in a 13-day culture

A larger number of more strongly luminescent cells can be seen than in Fig. 2. ×200.

Table III. CEA Content in Sera and in Tumors of Nude Mice Bearing Human Gastric Cancers

Cell line	Subcutaneous transplantation				Intraperitoneal transplantation			
	No. of tumor	Total tumor weight (g)	Serum CEA (ng/ml)	Tissue CEA (ng/mg)	No. of tumor	Total tumor weight (g)	Serum CEA (ng/ml)	Tissue CEA (ng/mg)
MKN28cl-4	1	2.1	11	1.6	1	1.5	83	3.6
	1	2.0	nd[a)]	2.0	3 (L)[b)]	1.5	18	4.0
	1	2.0	nd	4.7	3	1.3	117	2.3
	1	1.9	13	3.1	2	1.3	106	3.0
	1	1.8	nd	2.8	3 (L)	1.5	130	2.9
MKN45cl-42	1	2.7	142	28.4	2 (L)	1.1	286	52.6
	1	1.8	245	31.2	2	1.1	1750	73.2
	1	1.9	88	72.0	4 (L)	1.2	1793	105.6
	1	1.6	94	92.4	3	1.3	2288	63.2
	1	2.2	442	52.0	3 (L)	1.2	1420	54.3
KATO-III	1	1.1	11	nt[c)]				
	1	1.2	17	nt				
	1	1.1	8	nt				

a) nd: not detectable (<5 ng/ml).
b) (L): positive for liver invasion.
c) nt: not tested.

were heavier in subcutaneous heterotransplantation than in the intraperitoneal case. However, intraperitoneal inoculation resulted in the formation of two or three tumor masses in many cases. Liver invasion did not affect the serum CEA level (Table III). The subcutaneous and intraperitoneal transplantabilities of two cell lines, MKN-28cl-4 and MKN45cl-2, into nude mice were 100%. In the case of the KATO-III cell line, the subcutaneous transplantability was 18.8% (3/16), and intraperitoneal heterotransplantation failed completely (0/12).

Morphology and Localization of CEA in the Heterotransplanted Tumors in Nude Mice Tumors of MKN28cl-4 grown in nude mice became more anaplastic than the primary tumor. As for CEA localization, some cell clusters showed positive reaction mainly at the cell surface. These

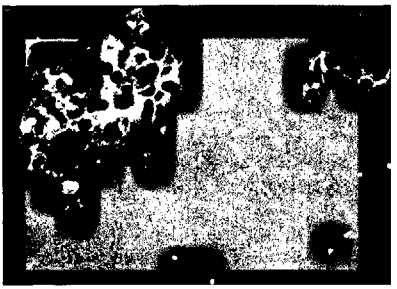

Fig. 4. Immunofluorescence micrograph of a transplanted tumor of MKN28cl-4 in a nude mouse. ×200

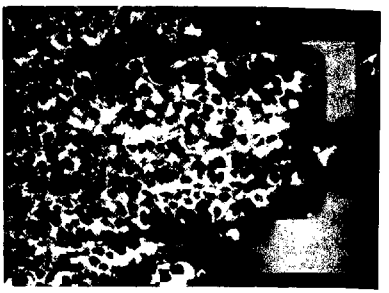

Fig. 5. Immunofluorescence micrograph of a transplanted tumor of MKN45cl-2 cells in a nude mouse

Many positively stained cells are apparent. However, there are also many negatively stained cells. Nodular proliferation is apparent. ×200.

Table IV. Frequency of CEA Positive Cells in Heterotransplanted Tumors in Nude Mice

Cell line	Positive cells[a] (%)	
	Subcutaneous transplantation	Intraperitoneal transplantation
MKN28cl-4	2.4±0.5	2.2±0.7
MKN45cl-2	47.8±12.1	51.4±10.9
KATO-III	0	nt[b]

a) Mean±SD (MKN28cl-4 and MKN45cl-2 n=5, KATO-III n=3).
b) nt: not transplantable.

cells were arranged partly in tubular structures (Fig. 4). There was no difference in the frequency of positively stained cells at the two sites of inoculation in either cell line, MKN28cl-4 or MKN45cl-2 (Table IV).

However, the positively stained cells in the tumor tissues derived from MKN45cl-2 were decreased in number in nude mice as compared with the *in vitro* situation. Negatively stained cells were found in considerable numbers and showed nodular proliferation (Fig. 5). Positively stained cells could not be detected by the indirect immunofluorescence method in the subcutaneous tumor tissues derived from KATO-III.

Discussion

CEA is often present in gastrointestinal tumor tissue in cases of well differentiated adenocarcinomas or poorly differentiated adenocarcinomas with mucin.[2, 3, 15] This tendency was confirmed in cultured gastric cancer cell lines. The production pattern of CEA, however, was cell line-dependent.

The CEA production in cell lines derived from well differentiated tubular adenocarcinomas was relatively low and dependent on the growth phase of the cells. The production began at the mid-exponential phase and reached its peak at the late stationary phase. The amount of CEA per 10^5 cells increased with culture time in these cell lines. Immunocytochemical studies revealed that the frequency of positively stained cells with anti-CEA sera was very low soon after plating but increased gradually with culture time. Therefore, in these cell lines, CEA productivity might be related to cell maturation.

On the other hand, the CEA production in cell lines derived from poorly differentiated adenocarcinomas and a signet-ring cell

carcinoma was high and was only slightly or was not dependent on the growth phase of the cells. In these cell lines, CEA was detected immediately after plating; the amount of the antigen per 10^5 cells was almost constant through the period of experiment and the frequency of positively stained cells with anti-CEA sera was very high even at the early exponential phase of cell growth and it did not increase significantly with culture time. These results suggest that CEA might be produced by a fixed population of cancer cells in these cell lines.

Intraperitoneal inoculation of two cloned gastric cancer cell lines produced high levels of serum CEA in the host mice. On the other hand, subcutaneous inoculation of them resulted in lower levels of CEA in the sera of the mice. However, the CEA contents and the frequency of CEA-positive cells in the tumor tissue grown from the inoculated clonal cell lines showed no significant difference between the two sites, subcutaneous or intraperitoneal. Thus, transport to the systemic blood flow of CEA produced by cancer cells *in loco* might be difficult after subcutaneous inoculation. The nature of the impediment to transport of CEA into the circulating blood in the subcutaneous region is not clear. The tumor mass might be the most important factor, but cannot account for all of the results. The vascularity of tumor tissues might not be a major factor, because the intensity of vascularization was only slightly more marked in the intraperitoneal than in the subcutaneous tissues, histologically. It was reported that the growth rates of human tumors growing in nude mice were influenced by the anatomical region in which they grew.[12] The appearance of CEA in sera of nude mice was also influenced by the anatomical location. In human cancers, it has been shown that there is no correlation between the CEA content of the cancer tissues and the CEA level in the serum.[10] These phenomena may also be related to the anatomical location of the tumor.

It has been suggested that CEA is metabolized in the liver[22] and it was reported that serum CEA levels were elevated in non-neoplastic liver diseases[16] or biliary tract obstruction.[13] In the heterotransplantation system, simple liver invasion by tumors grown in the hosts seemed not to hinder the clearance of CEA from the liver and did not influence the serum CEA level in the nude mice. Clinically, a rising CEA level may often reflect progressive hepatic dysfunction due to toxicity of chemotherapeutic drugs.[20]

Most MKN45cl-2 cells were CEA-positive *in vitro*. However, the CEA-positive cells were markedly decreased in number in nude mice. Human gastric cancer cells, especially those derived from poorly differentiated adenocarcinoma, may be unable to display their full potential for CEA production in nude mice.

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