Establishment of a New Human Gallbladder Carcinoma Cell Line (KMG-A) from an Alpha-Fetoprotein-Producing Gallbladder Carcinoma Transplanted into Nude Mice

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Summary: An alpha-fetoprotein (AFP)-producing human gallbladder carcinoma with direct invasion to the liver was transplanted into BALB/c-nu/nu nude mice and a new human gallbladder carcinoma cell line (KMG-A) has been established from the transplanted tumor. The patient's serum levels of AFP and carcinoembryonic antigen (CEA) were elevated at 1,040 ng/ml and 22.1 ng/ml, respectively after the operation. The tumor transplanted into nude mice was a poorly differentiated adenocarcinoma showing both solid and papillotubular structures. Serum levels of AFP, CEA, carbohydrate antigen 19-9 (CA 19-9), β2-microglobulin (BMG), and ferritin (FER), were elevated in the nude mice with tumor transplants. The KMG-A cell line has been maintained for 2 years through 80 passages. Morphologic features of KMG-A cells contained varying-shaped and-sized cells, including some multinuclear giant cells. KMG-A cells had one or more large round to oval nuclei with prominent nucleoli. The doubling time of cells grown in a serum-containing medium was about 28.5 hours and KMG-A cells were able to proliferate in a serum-free medium. Functionally, KMG-A cells produced CA 19-9, BMG, and FER, but AFP and CEA were not detectable in the supernatant of KMG-A cells. KMG-A cells were identified as containing AFP, CEA, CA 19-9, and albumin (ALB) by immunohistochemical techniques. The tumorigenicity of KMG-A cells was not identified by the subcutaneous inoculation of the cells into nude mice 4 months after inoculation, but histological examination of the subcutaneous tissue where the KMG-A cells were inoculated revealed small foci of poorly differentiated adenocarcinoma.

Key words: alpha-fetoprotein - alpha-fetoprotein-producing carcinoma - cell line - nude mouse - gallbladder carcinoma

Introduction

Alpha-fetoprotein (AFP) was first described as being secreted from an experimental liver tumor in a mouse by Abelev et al. (1963). In 1964, Tatarinov demonstrated the appearance of AFP in the serum of patients with hepatocellular carcinoma (HCC) and teratocarcinoma. Primarily, AFP is known as an important tumor marker, especially for HCC (O’Conor et al. 1970) and yolk sac tumor (Ballas, 1972). Although AFP is detectable in the serum of most patients with HCC, recent investigations have revealed that various other tumors produce AFP, including gastric carcinoma (Mori et al. 1970; Takagi et al. 1974; Kodama et al. 1981), lung carcinoma (Tsung 1975; Yasunami et al. 1981), pancreoblastoma (Iseki et al. 1986), gallbladder carcinoma (Hasebe et al. 1985; Iwase et al. 1986). Ezaki et al.
(1983) reported the establishment of an AFP-producing human gastric carcinoma in nude mice. However, gallbladder carcinoma is a less common disorder and the features of AFP-producing gallbladder carcinoma remain to be elucidated.

The present report describes the establishment and the characterization of a new human gallbladder carcinoma cell line from the AFP-producing gallbladder carcinoma transplanted into nude mice.

Materials and Methods

Case report

A 67-year-old male patient was diagnosed as having a gallbladder carcinoma which was directly invading into the liver. In June 1985, cholecystectomy and partial hepatectomy were performed. Before the operation serum levels of AFP and CEA were 9.0 ng/ml and 2.0 ng/ml, respectively, 4 months after the operation serum levels of AFP and CEA rose to 1,040 ng/ml and 22.1 ng/ml, respectively with spreading of liver metastasis. The patient died of liver failure 5 months after the operation; an autopsy was not performed.

Animals used and method of transplantation into nude mice

Four- to six-week-old female, athymic nude mice (BALB/c-nu/nu, Clea Japan Inc.) were used throughout the experiments. Part of the tumors obtained aseptically from the gallbladder and invaded portion of the liver were chopped and transplanted subcutaneously into the back of nude mice. When the transplanted tumors grew to as large as 20 mm in diameter, the tumors were used for tissue culture.

Tissue culture

An aseptically obtained specimen of a cancer nodule transplanted into nude mice was cut into 1- to 3-mm pieces in 60 mm dish (Falcon, Becton Dickinson Labware, Oxnard CA) filled with DMEMA + 20 % FCS [Dulbecco's modified Eagle medium (Nissui Seiyaku Co., Japan) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin ( Gibco, Chagrin Falls, Oh), 12 mM sodium bicarbonate, and 20 % fetal calf serum (M.A. Bioproducts, Walker-sville, MD)]. After removing red blood cells with a 20-μm pore mesh filter, small tissue fragments were washed in Ca- and Mg-free phosphate buffered saline (PBS) (Nissui), centrifuged (800 rpm 5 min at 4 °C), and the pellet was suspended in 20 ml of PBS with collagenase (150-250 unit/mg, WAKO Pure Chemical Industries, LTD., Japan) and incubated in a 37 °C water bath for 90 or 120 min. Thereafter, the fragments were filtered through 100 μm pore mesh to remove undigested tissue, and the cell suspension was centrifuged (800 rpm 5 min at 4 °C), resuspended in DMEMA + 20 % FCS, placed in 35- and 60-mm culture dishes, and cultured at 37 °C with 5 % CO2 in air. Undigested tissue fragments were also cultured in the same manner. The medium was renewed 2 to 3 times a week. After obtaining a stable cell growth, cells were cultured and maintained in T-25 flasks (Falcon) with DMEMA + 10 % FCS. For subculture, trypsin-EDTA (GIBCO) was used. Some cells were transferred for serum-free culture using chemically defined medium (RPMI 1640 Nissui) supplemented with antibiotics and selenium (Na2SeO4) (Nakabayashi et al. 1982).

Observations

The tumors transplanted into nude mice were fixed in 10 % formalin and embedded in paraffin. Tissue sections were examined with hematoxylin and eosin (H.E.), Mayer’s mucicarmine, and alcian blue stains. For electron microscopic observation, some of the tissue was fixed with Karnovsky’s fixing fluid, washed 3 times with 0.1 M sodium cacodylate buffer,
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postfixed in 1% osmium-millonig, dehydrated in graded ethanol, and embedded in Epon 812. Ultrathin sections cut on a LKB ultratome were double-stained with uranyl acetate and lead citrate and were observed by a HITACHI H-500 electron microscope.

KMG-A cells were inspected daily by phase contrast microscopy (Nikon, Japan). For light microscopic observation, cells grown on Lab-Tek tissue culture chamber slides (Miles Laboratories, Naperville, IL) were washed with PBS three times, fixed in 95% ethanol or absolute methanol for 20 min, then stained with H.E., Mayer's mucicarmine, and alcian blue stains. For electron microscopic observation, cells grown on Lab-Tek tissue culture chamber slides were treated as previously described (Murakami 1984; Yano et al. 1986; Murakami et al. 1987) and examined under a HITACHI H-500 electron microscope.

**Immunohistochemical studies**

Localization of AFP, CEA, ferritin (FER), fibrinogen (FIB), albumin (ALB), and carbohydrate antigen 19-9 (CA 19-9) in the tumor transplanted into nude mice were examined using the avidin-biotin peroxidase complex (ABC) method (ABC kit, Vector Laboratories, Inc. Burlingame CA) (Kakizoe 1987). KMG-A cells grown on Lab-Tek tissue culture chamber slides were washed as previously described (Murakami 1984; Yano et al. 1986; Murakami et al. 1987) and examined under a HITACHI H-500 electron microscope.

**Tumor markers**

The sera of nude mice with transplanted tumor was assayed for AFP, FER, CEA, β2-microglobulin (BMG) and CA 19-9 by means of radioimmunoassay kits. The tumor markers secreted by KMG-A cells for 48 hours in 5 ml serum-containing medium were examined. The spent media were centrifuged (3,000 rpm 10 min at 4°C), and the supernatants were assayed for AFP, CEA, FER, BMG, and CA 19-9 in the same manner (AFP, CEA: Dinabot, Japan; FER: Clinical assays, Cambridge, MA; BMG: Eiken Immunochemical Lab., Japan; CA 19-9: Centocor, USA).

**Chromosome study**

KMG-A cells in the 17th passage were used for chromosome studies. Four days after seeding the cells in the T-25 flask, colcemid was added to the flask (0.04 μg/ml) and incubated at 37°C for 2 to 4 hours. Cells were detached with 0.25% trypsin, and treated with hypotonic (0.075 M) KCl for 10 min, centrifuged and fixed with a mixture of methanol and acetic acid (3:1) twice. The cells suspended in fixative were dropped on slides, and flame dried, and stained with Giemsa. G-banding was carried out using the method of Seabright (1971) by staining the cells with Giemsa after 5 min 0.0125% trypsin treatment.

**Heterologous transplantation of KMG-A cells**

Four- to six-week-old female athymic nude mice were used to examine the tumorigenicity of KMG-A cells. Fifteen million and 40 million cells at 20th passage suspended in 300 μl DMEMA + 10% FCS were inoculated subcutaneously into the back of two mice. The mice were killed after 4 months and their tissue was observed by light microscopy.

**Mycoplasma detection**

Mycoplasma contamination was examined by DNA fluorochrome staining with
Mycoplasma Detection kit (Bioassay Systems Corp., Woburn, MA). KMG-A cells grown on Lab-Tek tissue culture chamber slides were fixed with absolute methanol 20 min at 37 °C. The specimens were examined with fluorescence microscopy at ×400 under oil immersion.

Results

Morphology of the tumor transplanted to nude mice

The transplanted tumor exhibited both solid and glandular structures. In the area showing a solid structure, tumor nests consisted of polygonal cells with bizarre nuclei and prominent nucleoli, separated by thin fibrous stroma (Fig. 1A). The area showing glandular structure had various-sized tumorous glands packed closely together with little intervening stroma (Fig. 1B). Many mitotic cells were found in both areas. Mucin could be detected in the lumen of the tumorous glands by PAS, mucicarmine, and alcian blue stains. The histologic features of the transplanted tumor did not change after serial transplantations. By electron microscopic examination, numerous microvilli were observed on the cell surfaces. Some of the tumor cells had mucus droplets (Fig. 2).

The presence of AFP, CEA, FER, CA 19-9, and ALB were observed in both areas of solid and glandular patterns (Fig. 3). FIB-positive cells were not seen in the foci of the glandular pattern but were observed in the foci of the solid pattern.

Establishment and morphology of KMG-A cell line

A few days after primary culture, some colonies of epithelial-like cells were observed in several dishes. Under phase contrast microscopy, these colonies increased in size, and piling up of these cells in the center of the colonies, suggesting that they lack contact inhibition, was observed. KMG-A cell line was initially established from a 60-mm dish at the 12th day. After scratching out fibroblast-like cells surrounding the colonies with needles, these colonies were transferred to 24-well, flat-bottom plates (Falcon) by treatment with trypsin-EDTA, and then subcultured to T-25 flasks, successively. Subsequently, four cell lines that were morphologically and functionally similar each other from different four dishes were successively obtained. Since this cell line grows continuously and has been serially passaged 30 times over 8 month-period, it is designated KMG-A.

KMG-A cells, grown in a serum-containing medium, showed various in shape and size, consisting of polygonal, spindle, round, and multinuclear giant cells, and had one or more prominent nucleoli. They exhibited a typical epithelial feature, namely a pavement-like cell arrangement. Some cells contained intracytoplasmic, mucicarmine-positive materials (Fig. 4).

By electron microscopy the characteristics of these cells were abundant microfilaments and mitochondria in the cytoplasm and prominent nucleoli (Fig. 5).

Various intensities of CEA-, CA 19-9-, AFP-, and ALB-positive reactions were identified in the cytoplasm of KMG-A cells. The distribution pattern of CA 19-9-positive cells was more diffuse than that of other antigen-positive cells and showed a mosaic pattern.

Growth kinetics of KMG-A cells

The growth characteristics of KMG-A cells at 22nd passage grown in a serum-containing medium revealed that the cells grew rapidly after lag phase for 48 hours and entered a logarithmic growth phase (Fig. 6). The doubling time of the cells in a serum-containing medium was 28.5 hours. The saturation density was approximately 15.6 × 10^4 cells/cm^2. KMG-A
A) The area showing solid pattern. H.E. ×50.

B) The area showing glandular structure. H.E. ×50.

Fig. 1. Histologic features of the transplanted tumor.
Fig. 2. Electron microscopic finding of the transplanted tumor. Note the well-developed microvilli and mucus droplets. ×3375.

Fig. 3. Localization of AFP in the transplanted tumor. ABC method: ×100.
Fig. 4. Light microscopic finding of KMG-A cells showing pavement-like structure, with multinuclear giant cells. Inset: Mucicarmine-positive material (arrow head) in the cytoplasm of the cell. H.E. stain ×100; inset Mayer's mucicarmine stain ×200.

Fig. 5. Electron microscopic finding of KMG-A cells. Note abundant mitochondria and well-developed microfilaments. ×5000.
cells are also able to grow in a serum-
free medium, but required long adaptation
periods and grew slowly.

**Tumor markers**

The patient's serum AFP, CEA, and
CA 19-9 levels rose 4 months after the
operation to 1,040 ng/ml, 22.1 ng/ml, and
5 months after the operation to 280,000 U/
ml, respectively. The maximum serum
levels of tumor markers in nude mice were
as follows; AFP, 1,080 ng/ml; CEA, 1,020
ng/ml; CA 19-9, 150,000 U/ml; BNG, 650
µg/l; FER, 240 ng/ml. KMG-A cells

![Fig. 6. Growth curve of KMG-A cells in a serum-containing medium at the 22nd passage. Doubling time is approximately 28.5 hours.](image)

![Fig. 7. Correlation between CA 19-9 secretion and growth curve.](image)

![Fig. 8. Distribution of chromosome number at the 17th passage of KMG-A cells.](image)
TABLE 1

Concentration of AFP, Ferritin, BMG, CEA and CA 19-9 in sera of
patient and NuKMG and spent media by KMG-A

<table>
<thead>
<tr>
<th></th>
<th>Patient</th>
<th>NuKMG</th>
<th>KMG-A</th>
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<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>c</td>
</tr>
<tr>
<td>AFP (ng/ml)</td>
<td>9.0</td>
<td>1040</td>
<td>23500</td>
</tr>
<tr>
<td>Ferritin (ng/ml)</td>
<td></td>
<td></td>
<td>2500</td>
</tr>
<tr>
<td>BMG (μg/l)</td>
<td></td>
<td></td>
<td>120</td>
</tr>
<tr>
<td>CEA (ng/ml)</td>
<td>2.0</td>
<td>22.1</td>
<td>37.5×10⁴</td>
</tr>
<tr>
<td>CA 19-9 (U/ml)</td>
<td></td>
<td></td>
<td>28×10⁴</td>
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a : 2 days pre-operatively  
b : 4 months post-operatively  
c : 5 months post-operatively  
NuKMG : nude mice with transplanted tumor

Fig. 9. A G-banded karyotype at the 17th passage of KMG-A cells with 68 chromosomes.
grown in a serum-containing medium secreted CA 19-9 (2,800 U/ml), BMG (180 μg/l), FER (6.1 ng/ml). AFP and CEA were not detectable in the spent media (Table 1).

With respect to the correlation between cell growth and the secretion of CA 19-9 in the spent media, CA 19-9 was identified in any particular phase in parallel with the growth curve. The concentration of CA 19-9 per 10^4 cells increased in parallel with the growth curve and reached a maximum level 12 days after seeding (Fig. 7).

**Chromosome study**

The number of chromosomes was distributed in the range 61 to 72 without an obvious mode, but was mainly distributed between 65 and 69 (Fig. 8). G-band analysis revealed human karyotype and several structural abnormalities of cells, including 1q-, 4q+, 7q+, 7q-, and No. 8 monosomy, with the emergence of marker chromosomes. The G-band karyotype of the cell containing 68 chromosomes is shown in Fig. 9.

**Heterologous transplantation**

No visible tumor developed within 3 to 4 months in any nude mice subcutaneously inoculated with KMG-A cells, but at necropsy a small tumor nest was observed microscopically in the subcutaneous tissue where KMG-A cells had been inoculated. Histopathologically, subcutaneous tumor showed a poorly differentiated adenocarcinoma. The tumor cells contained large atypical nuclei with one or two prominent nucleoli (Fig. 10).

**Mycoplasma detection**

Mycoplasma contamination was negative in KMG-A cells.

![Fig. 10. Histologic finding of the subcutaneous tumor observed in the back of a nude mouse. The tumor shows a solid growth pattern. H.E. ×100.](image-url)
Discussion

AFP is a glycoprotein with a molecular weight of 70,000 daltons, containing about 4 % carbohydrate (Ruoslahti and Seppälä 1971), and is known as an embryonal serum protein produced primarily by the fetal liver and yolk sac and, to a lesser extent, by the gastro-intestinal tract (Gitlin et al. 1972). AFP was initially thought to be specific for HCC, yolk sac tumor and teratoma. However, the development of radioimmunoassay permitted recent investigations to reveal that various levels of AFP were detectable in the serum of patients with many other kinds of tumor, including gastric, colonic, pancreatic, and biliary carcinomata (Kato et al. 1974; McIntire et al. 1975). High levels of AFP in the sera of patients with gastric, pancreatic, or biliary tract carcinoma were found with a greater incidence than that with carcinomata of colon, esophagus, and small bowel (McIntire et al. 1975).

It is interesting that some of AFP-producing tumors show histopathologic and/or functional hepatoid features, such as intracytoplasmic and extracytoplasmic PAS-positive hyaline bodies, and the presence of alpha-1-antitrypsin, ALB, and prealbumin (Plat et al. 1982; Ishikura et al. 1985). Ishikura et al. (1986) proposed a new entity of the gastric carcinoma, which was named “hepatoid adenocarcinoma of the stomach” and characterized by high level of serum AFP, histopathological features closely similar to HCC, and Concanavalin A-property of hepatic type; this carried a poor prognosis because of frequent liver metastasis. Concerning poorly differentiated adenocarcinoma of the stomach of the medullary type, Sawada (1986) reported that the tumors showed a high morbidity rate with early hepatic metastasis and the presence of AFP-positive cells in some of the tumors. The poor prognosis of AFP-producing tumors might be attributed to an immunosuppresive effect of AFP (Yachnin, 1983).

The present work describes the successful establishment of a new human gallbladder cell line (KMG-A) from an AFP-producing carcinoma transplanted into nude mice. A few cell lines of human gallbladder carcinoma have been established (Morgan et al. 1981; Knuth et al. 1985; Egami et al. 1986). Knuth et al. (1985) reported three new human cell lines from adenocarcinoma of the extrahepatic biliary tract, one of which secreted AFP into the tissue culture supernatant. In the present experiments, KMG-A cells produced CA 19-9, BMG and FER, but AFP and CEA were not detectable in the spent media. Immunohistochemically, positive reactions for AFP, CEA, FER, and ALB were identified in KMG-A cells. This fact suggests that the capacity of KMG-A cells to synthesize AFP was retained. The reasons why AFP was not detectable in the spent media of KMG-A cells are considered to be as follows: 1) the total number of cells in the flask was much smaller than that of tumor transplanted to nude mice; 2) KMG-A cells could synthesize AFP, but could not secrete it into the supernatant; 3) non-AFP-producing cells were selectively cultured.

Regarding the secretion of AFP by cultured cancer cells, the regulation of AFP secretion and growth rate by various hormones in established human HCC cell lines were recently studied (Nakabayashi et al. 1985). These cell lines were regulated positively or negatively by some hormones. Yano (1986) demonstrated that a human HCC cell line secreted AFP only in a serum-free medium and not in a serum-containing medium. KMG-A cells could proliferate in a serum-free medium but the growth was very slow and AFP was not detectable in the spent media.

To confirm the AFP synthesis and the tumorigenicity of KMG-A cells, KMG-A
cells were inoculated subcutaneously into the backs of nude mice (15 × 10^6 or 40 × 10^6 cells/mouse in a single site). Although a visible tumor mass could not be found, histologic examination of the subcutaneous tissue, where the cells were inoculated, revealed small foci of poorly differentiated adenocarcinoma. The reasons why KMG-A cells derived from the transplanted tumor to nude mice did not form the obvious tumor in nude mice are considered to be as follows: 1) KMG-A cells lost tumorigenicity during the culture process, 2) the number of the inoculated KMG-A cells was not adequate for the examination of tumorigenicity.

Abnormalities involving chromosome 1, which are most frequently observed in solid tumor malignancies (Kakita et al. 1975, Cruciger 1976, Morgan et al. 1981), were detected in KMG-A cells. Double minutes, which were one type of abnormal chromosomes, reported by Morgan et al. (1981) in a human gallbladder carcinoma cell line were not observed in KMG-A cells. It is evident that AFP gene is located on chromosome 4, and the emergence of an abnormality of chromosome 4, seen in all KMG-A cells examined, might be related to AFP secretion of KMG-A cells.

After 2 years in culture, KMG-A cell line maintains a distinct epithelioid morphology and continues to secrete several tumor markers. This KMG-A cell line is available to other qualified investigators for their studies.

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