Establishment of a Human Urachal Adenocarcinoma Cell Line (KO-BT-1) and Its Chemosensitivity

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ABSTRACT
A new human urachal adenocarcinoma cell line (KO-BT-1) was established and characterized. It consisted of cuboidal, spindle, and polymorphic giant cells and continued to grow for more than 27 months without contact inhibition. Doubling time was about 15.5 h at the 70th passage. In nude mice the cells produced tumors, the histologies of which were similar to the original patient-derived tumor. Moreover, serum carcinoembryonic antigen level was elevated in the patient and the histological section of tumor formed in nude mice was stained with an antibody to carcinoembryonic antigen by peroxidase-antiperoxidase method. Electron microscopically, the cells covered with microvilli had cell-junction complexes. The chromosome number was aneuploid with a modal number of 60. At clinically achievable concentrations, doxorubicin, cis-platinum, mitomycin C, and 40487S which was an in vitro-active type of cyclophosphamide did not reduce colony formation to 30% or less of the control value; likewise in this patient chemotherapy had not been effective. In addition, among human recombinant tumor necrosis factor, α-interferon, and recombinant β- and γ-interferon, recombinant IFN-β was most effective against this tumor. These results indicated that KO-BT-1 cells showed the similar chemosensitivity and properties to those of the original tumor, and might be useful in basic studies for the diagnosis, treatment, and etiology of urachal tumors.

INTRODUCTION
Of all malignant neoplasms found in urogenital lesions, urinary bladder tumor is the most frequent, and among these neoplasms histologically transitional cell carcinoma is very common. Although urachal carcinoma is a comparatively rare malignant neoplasm (1-3), making up only 0.17-0.34% of primary urinary bladder tumors, and 20-39% of primary bladder adenocarcinomas, it is most commonly seen in the fourth decades and has a poor prognosis (2). Most urachal cancers are either mucin-producing (69%) or nonproducing (15%) adenocarcinomas (2). Its etiology, differential diagnosis to distinguish between primary bladder adenocarcinoma and urachal carcinoma, and treatment are as yet uncertain (2, 3).

Although some long-term cultured cell lines have been established from urinary bladder cancer, such as RT4, T24, J82, and HT-1197, they are histologically transitional cell carcinomas (4). To date there has been only one long-term cultured cell line of urachal adenocarcinoma (5). But we have now established another and have undertaken its characterization as a first step in studying the etiology, diagnosis, and treatment for the disease.

Recently, many investigators reported that TNF3 and IFN were effective against human malignant neoplasm (6, 7). We investigated their antineoplastic activities against our newly established cell line to study more effective chemotherapy.

This paper details the in vitro characterization and chemosensitivity of our new human urachal adenocarcinoma cell line (KO-BT-1).

MATERIALS AND METHODS
Tumor Origin. KO-BT-1 was derived from an adenocarcinoma of the urinary bladder (urachal tumor) of a 34-year-old man whose serum CEA level was elevated (125 ng/ml; normal range was less than 5 ng/ml). On March 6, 1982, partial cystectomy was performed. After 33 months, he was readmitted to the hospital due to tumor recurrence. Though chemotherapy combined with ADM, CDDP, MMC, 5-FU, and CMF was performed, his condition worsened. He died of carcinomatosis on May 25, 1985.

Tissue Culture. Before the chemotherapy, metastasized tissue was removed from the right inguinal lymph node, and was histologically shown to be an urachal adenocarcinoma metastasis. This tissue was used for in vitro culture on November 20, 1984. One piece was aseptically cut into small fragments and enzymatically dissociated for 2 h at 37°C with dispase (1000 units/ml; Gohdoh Shusei Co., Tokyo, Japan), and cell fragments were enzymatically dissociated for 2 h at 37°C with dispase (1000 units/ml; Gohdoh Shusei Co., Tokyo, Japan). The tumor-bearing mice were later sacrificed for histological examination and the tumor was stained with H&E, Giemsa solution and macroscopically counted.

The resulting tumor cell suspension was passed through a No. 100 steel mesh, was washed twice with DMEM, and was placed in 60-mm plastic Petri dishes (Falcon Plastic Corp., Oxnard, CA) in MEM containing 10% FBS, penicillin G (100 units/ml), and streptomycin (100 µg/ml) (Grand Island Biological Co., Grand Island, NY). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. At the 10th passage, the growth medium was changed to DMEM. Cells were subcultured by treatment for several minutes with 0.1% trypsin, and were conservatively diluted by only 1:3 to 1:6 every 7 days. Cells were treated with H&E, PAS reagent, and alcain blue stain.

Growth Curves. Cells were plated at a density of 1 x 105 viable cells/35-mm plastic Petri dish (Falcon Plastic Corp.) in 2 ml DME and were then cultured at 37°C in a 5% CO2 incubator. Every day cells in two tissue culture dishes were harvested and counted, viability being assessed by trypan blue exclusion. The average number of nonstained cells per dish at each time point was plotted.

Plating Efficiency. To study the PE of cells on plastic surfaces, cells at passage 40 were dispersed into a single cell suspension, and were plated onto 60-mm plastic Petri dishes at varying densities in 5 ml of DME. After 12 days, the colonies formed were stained with 10% Giemsa solution and macroscopically counted.

The ability of tissue culture cells to grow in soft agar was assayed using the double-layer soft agar system described by Salmon and Hamburger (8, 9). Aggregates composed of more than 40 cells were counted as colonies under the inverted microscope after 21 days' incubation at 37°C. Both on plastic surfaces and in soft agar, the PE was calculated as the number of colonies formed/the number of viable cells plated per dish. Each experiment was performed on triplicate dishes and repeated twice.

Transplantation of Cells into Nude Mice. A total of 1 x 105 viable cells at passages 35 and 65 were transplanted s.c. into the backs of five 7- to 8-week-old BALB/c nu/nu nude mice (Curea Laboratory Animal Center, Osaka, Japan). The tumor-bearing mice were later sacrificed for histological examination and the tumor was stained with H&E, PAS, alcain blue, and an antibody to CEA by the peroxidase-antiperoxidase method.
Fig. 1. Phase contrast photomicrograph of KO-BT-1 cells at passage 50 (x 250). A, cuboidal cells; B, spindle cells; C, polymorphic giant cells. The cells had large and prominent nucleoli, and showed loss of contact inhibition of growth.

Table 1 Colony-forming ability of KO-BT-1 cells

Each experiment was performed on triplicate dishes and repeated twice.

| No. of viable cells plated | No. of colonies on plastic surfaces | No. of colonies in semisolid agar
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<td>First experiment</td>
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<tr>
<td>1 x 10⁴</td>
<td>31 ± 5</td>
<td>28 ± 7</td>
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<td>1 x 10⁵</td>
<td>260 ± 18</td>
<td>285 ± 30</td>
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<tr>
<td>3 x 10⁶</td>
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* Singly dispersed cells were plated in 60-mm plastic Petri dishes and then cultured for 12 days at 37°C in a 5% CO₂ incubator. After staining with Giemsa solution viable colonies formed were counted.

* Cells were mixed with 1 ml 0.3% agar medium and then overlaid onto a basal layer of 1 ml 0.5% agar medium in 35-mm plastic dishes. After 21-day incubation at 37°C the colonies formed were counted.

* Values, mean ± SD of the values obtained from triplicate dishes.

* NT, not tested.

Carnoy's solution. The chromosome specimens were prepared by the ordinary air-drying method. Preparations were scored for the number of chromosomes present in each of 20 metaphase cells.

Electronmicroscopic Study. Cultured cells harvested by scraping with a rubber policeman were fixed for 1 h in 2.5% glutaraldehyde buffered with 0.1 M PBS (pH 7.3) containing 5 mM calcium chloride. These cells were washed twice with PBS and then post fixed with 2% osmium tetroxide for 1 h. They were dehydrated with stepwise increasing concentrations of ethanol and then embedded in Epon 812.

Heterotransplanted nude mouse tumors, after being minced into small pieces, were fixed in 4% glutaraldehyde in phosphate buffer for 2 h, and then processed by the same method as that used for the cultured cells. Thin sections cut on an ultramicrotome were stained with uranyl acetate and lead hydroxide, and were observed under an electron microscope (JEM 1200EX; Nippon Densi, Tokyo, Japan).

Chemosensitivity to Anticancer Drugs. We studied cell chemosensitivity to five anticancer drugs (ADM, CDDP, MMC, 5-FU, and 40487S which is an in vitro-active type of CMF) and four new antineoplastic agents [rHu-TNF (10), Dai Nippon Pharmaceutical Co., Osaka; IFN-α (11), Sumitomo Pharmaceutical Co., Osaka; and rec-IFN-β (12) and rec-IFN-γ (13), Kyowa Hakko Co., Tokyo] using the in vitro clonogenic assay. Cells in the logarithmic growth phase were seeded at 5 x 10⁴ viable cells/60-mm plastic Petri dish, were treated with each of the standard drugs at various concentrations including 10% of the peak plasma level achievable in humans (9, 14) (ADM, 0.04, 0.4, and 1.0 μg/ml; CDDP, 0.02, 0.2, and 1.0 μg/ml; MMC, 0.01, 0.1, and 1.0 μg/ml; and 40487S, 3.0, 30, and 300 μg/ml for 1 h at 37°C, and were then...
Fig. 3. A, KO-BT-1 (passage 35) tumor from a nude mouse. (H&E × 250); B, urachal tumor from which KO-BT-1 was derived (H&E × 250). Both tumors were mucin-producing adenocarcinomas. Some mitotic figures are visible.

washed twice with growth medium. The cells were subsequently cultured in DMEM containing 20% FBS, penicillin G (100 units/ml), and streptomycin (100 µg/ml) for 12 days. In some experiments, rHu-TNF, IFN-α, rec-IFN-β, rec-IFN-γ (each at 10, 100, and 1000 units/ml), and 5-FU (1.0, 10, and 100 µg/ml) were added to the culture medium with the cells. Total volume of the medium was exchanged with the same volume of fresh medium containing the drug every 3 days, for 12 days. The colonies formed were stained with Giemsa and then counted. The assay was performed in triplicate dishes at each drug concentration, and was repeated at least three times. Cells were considered to be “sensitive” to the drug, when the colony formation was reduced to 30% or less of the control value (9, 14).

RESULTS

Establishment of KO-BT-1 Cell Line. Many islands of epithelial cells were observed in primary culture 3 days after plating. The epithelial cells and fibroblasts grew to confluent monolayer sheets, and a half volume of the medium was changed every 3 to 4 days. Cells were subcultured at a dilution of 1:2 to establish
the secondary culture on the 60th day. In the first few early passages, some contaminating fibroblasts were observed, but they disappeared gradually during culture in MEM containing d-valine. From the 10th passage onwards, about 180 days after the primary culture, only epithelial cells continued to grow. The cell line thus obtained, KO-BT-1, was found to be adherent epithelial, composed of cuboidal, spindle, and polymorphic giant cells (Fig. 1A–C), with large nuclei and prominent nucleoli and cytoplasmic granules which stained with PAS and alcian blue. The cells have been continuously cultured for more than 27 months, have been passed 92 times and show loss of contact inhibition of growth.

Cell Growth. KO-BT-1 cells demonstrated rapid growth in vitro. In two experiments using cells at different passages (20th and 70th), the doubling times were approximately 48 h and 15.5 h, respectively. When cultured in growth medium containing various concentrations of FBS, 10% and above supported cell growth equally well as 20%, while 5% or less caused growth retardation. In serum-free medium, the cells survived, but did not attach to the plastic surfaces and did not grow for the first 3 days after plating; after four days of incubation the number of the cells gradually decreased (Fig. 2).

Plating Efficiency. KO-BT-1 cells formed colonies both on plastic surface and in soft agar. The number of colonies formed are shown in Table 1. The PEIs were 0.28 (28%) and 0.006 (0.6%) on plastic and in soft agar, respectively.

Tumorigenicity. Within about 60 days after inoculation of a total number of $1 \times 10^7$ viable tumor cells, a tumor mass had developed to the size of 10–15 mm in diameter. The tumors in nude mice were histologically poorly differentiated mucinous adenocarcinomas, which stained with PAS and alcian blue and had cytoplasmic granules stained with an antibody to CEA. They were similar to the original tumor (Fig. 3, A and B).

Chromosome Count. KO-BT-1 had a modal chromosome number of 60. KO-BT-1 was characterized by aneuploidy, the number of chromosome aberrations ranging from 39 to 102 (Fig. 4).

Electronmicroscopic Study. Both KO-BT-1 cells and the nude mouse tumor cells contained organelles (rough endoplasmic reticulum and mitochondria) in glycogen-rich cytoplasm and had cell-junction complexes. The cells were lined by numerous microvilli (Fig. 5).

Chemosensitivity to Anticancer Drugs. The three drugs except of 5-FU and 40487S, and the new antineoplastic agents inhibited colony formation by KO-BT-1 in a dose-dependent manner. At a concentration in the medium equal to 10% of the peak plasma level achievable in humans (ADM, 0.04 µg/ml; CDDP, 0.02 µg/ml; and MMC, 0.01 µg/ml), none of the standard drugs reduced colony formation to 30% or less of the control value. At the maximum concentration tested, higher than that clinically achievable, the inhibitory effects on colony formation were 100, 60, and 80% for ADM, CDDP, and MMC, respectively.
Fig. 6. Chemosensitivity of KO-BT-1 to anticancer drugs. A, percentage of surviving colonies of KO-BT-1 cells (70th passage) following exposure to increasing concentrations of ADM (○), CDDP (●), and MMC (□) for 1 h at 37°C. B, percentage of surviving colonies following exposure to increasing concentration of 5-FU (○) for a period (12 days), and 40487S (●), which is an in vitro active type of CMF for 1 h at 37°C. C, cells incubated for a period (12 days) with rHu-TNF (○), IFN-α (□), rec-IFN-β (●), and rec-IFN-γ (△) alone. At a concentration in the medium equal to 10% of the peak plasma level achievable in humans (ADM, 0.04 μg/ml; CDDP, 0.02 μg/ml; MMC, 0.01 μg/ml; 5-FU, 1.0 μg/ml; and 40487S, 3.0 μg/ml) reflecting the concentrations of standard drugs in tumor tissue, none reduced colony formation to 30% or less of control value. At clinically achievable concentrations of the new antineoplastic agents (rHu-TNF, 100 units/ml; IFN-α, 100 units/ml; rec-IFN-β, 100 units/ml; and rec-IFN-γ, 100 units/ml), rec-IFN-β reduced colony formation to less than 30% of the control value. The percentage of surviving colonies was measured after 12 days of culture (average of three Petri dishes).

DISCUSSION

Urachal carcinoma is an uncommon neoplasm, with an incidence of 0.17–0.34% of all bladder cancers (1). The prognosis is poor (1–3), with only 6–12% of patients showing a 5-year survival rate (16).

To date, many long-term cultured cell lines of human urinary bladder cancers have been established (4), but there has been only one urachal adenocarcinoma cell line (5). Our new human urachal adenocarcinoma cell line (KO-BT-1), histologically a mucinous adenocarcinoma, was isolated in our laboratory from an inguinal lymph node-resident metastasis. It was proved that this was a malignant cell line of epithelial origin by the following findings: (a) the cells had an epithelial morphology and exhibited no contact inhibition of growth as demonstrated by cell multilayering; (b) this cell line when inoculated into nude mice was able to produce tumors whose histological appearance was quite similar to that of the original tumor; (c) the tumor formed in nude mice had cytoplasmic granules stained with an antibody to CEA by the peroxidase-antiperoxidase method; (d) karyotypic analysis revealed an abnormal chromosome number; (e) the cells remained in continuous culture for more than 27 months without any evidence of a decreased growth rate; (f) electronmicroscopic observations revealed a poorly differentiated adenocarcinoma similar to adenocarcinoma of other organs and the cells had cell-junction complexes (desmosome) (17); and (g) the cells produced colonies in soft agar.

One difficulty in establishing cell lines from human tumors is that stromal fibroblasts frequently grow with the malignant cell population and tend to overgrow the culture. There are several methods, such as differential centrifugation, differential trypsinization, and physical removal of colonies, to eliminate the fibroblasts, but Gilbert et al. (18) showed that they could not grow in culture medium without L-valine. Thus we used MEM containing D-valine instead, and FBS which had been dialyzed against PBS (pH 7.4) for 3 days at 4°C. Under these conditions fibroblastic cells gradually died, and only epithelial-like cells continued to grow. At passage 10, we changed the culture medium to DMEM with FBS, since by this time the cells were composed of only epithelial-like cells. At passage 25, spindle-like cells appeared and they were presumed to be a subclone of the epithelial cells since they continued to grow and pile up for more than 15 months and had cytoplasmic granules stained with PAS and alcian blue.

The KO-BT-1 cells were resistant to anticancer drugs (ADM, CDDP, MMC, and 40487S) in vitro, and likewise the patient’s clinical response to treatment, which was performed after resection of the lymph node, with the same drugs was poor. This indicated that the cells in vitro had a chemosensitivity similar to the tumor cells of the patient. Although, in treatment by 5-FU the clinical response was poor, the cells were sensitive to the drug in vitro. As the doubling time of cell growth was shorter than that of the original tumor, 5-FU was considered to be more effective against the cells than against original.
tumor. Moreover, the finding that KO-BT-1 cells were sensitive to 5-FU and had cytoplasmic granules stained with an antibody to CEA indicated they were similar to adenocarcinoma. Recently, many investigators reported that TNF and IFN were effective for human malignant tumors (6, 7). We investigated the chemosensitivity of KO-BT-1 to rHu-TNF, IFN-α, rec-IFN-β, and rec-IFN-γ alone. Against the cells, rec-IFN-γ was a more effective agent than the other drugs, indicating that it might be useful in the treatment of urachal carcinoma.

Tveit et al. (19) demonstrated that cell lines might not accurately reflect the biological characteristics of the original neoplasm, but it was demonstrated in this study that KO-BT-1 did have comparatively similar properties to its original tumor. Thus, this cell line should be useful in basic studies of the diagnosis, treatment, and etiology of the disease.

REFERENCES

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