Sex Hormone Response of a Newly Established Squamous Cell Line Derived from Clinical Esophageal Carcinoma

Hideo Matsuoka, Keizo Sugimachi, Hiroaki Ueo, Hiroyuki Kuwano, Shuji Nakano, and Masaaki Nakayama

Department of Surgery II [H. M., K. S., H. U., H. K.] and First Department of Internal Medicine [S. N., M. N.], Faculty of Medicine, Kyushu University, Maidashi, Higashi-ku, Fukuoka 812 Japan

ABSTRACT

Biopsy tissues from a 68-year-old Japanese man with metastases to axillary lymph nodes of a recurrent esophageal carcinoma were adapted to cell culture conditions and a continuously growing tumor cell line was developed. Immunohistochemical staining revealed that these cells contained keratinous material and the electron microscopic study revealed the presence of tonofilaments. Thus, this line, designated the KSE-1 line, was considered to have originated from metastatic squamous cell carcinoma of the esophagus. This line has a binding content of 4.2 fmol/mg protein for the estrogen receptor and 2.2 fmol/mg protein for the testosterone receptor. By measurement of cell number and thymidine incorporation, the growth rate of this line was found to be moderately responsive to these hormones, being inhibited by estrogen and enhanced by testost.

INTRODUCTION

Esophageal carcinoma occurs more frequently in men, the ratio being 2:1 to 20:1 (1, 2), and the postoperative course is generally more favorable in women (3). Sugimachi et al. (4) found that DNA distribution patterns measured in Feulgen-stained sections, using the two-wavelength method, is a reliable prognostic factor. They showed that biological differences between esophageal carcinoma in males and females might be due to differences in such DNA distribution patterns (5). These findings suggested that growth of the esophageal cancer cells and the prognosis of the patient might be influenced by hormones, such as testosterone and/or estrogen. Moltjen et al. (6) reported that several head and neck tumors, including squamous cell carcinoma of the maxilla, presented with high levels of estrogen receptor protein. Although epithelial cell lines derived from a human esophageal cancer have been described (7, 8), there seems to be no report on the binding contents for sex hormone receptors and characterization with regard to control of growth by sex hormones. We established a human esophageal carcinoma cell line and made use of this line to determine relative biological and histological properties. Particular attention was directed to the binding contents for both estrogen and testosterone receptors.

MATERIALS AND METHODS

Chemicals. EMEM without phenol red was purchased from Nissui Pharmaceutical Co., Ltd. FCS was obtained from GIBCO Oriental Co., Ltd. Sodium piperacillin was purchased from Toyama Chemical Co., Ltd., Japan. Gentamicin was from Schering Co. Amphotercin B was obtained from GIBCO Oriental Co., Ltd. Dextran T-70 was obtained from Pharmacia, Uppsala, Sweden. [3H]dThd and [1,2,6,7-3H]DHT were purchased from Amer sham. 16α-17β-estradiol was obtained from Otsuka Assay Laboratories, Japan.

Source of Tumor Specimen. A 68-year-old Japanese man was diagnosed as a case of carcinoma of the middle esophagus, and an esophagectomy was done on September 17, 1979. In February 1985, there was a recurrence in the axillary lymph nodes. A biopsy was performed and a part of the specimen was used for the primary cultures.

Primary Culture and Establishment of KSE-1 Cells. The viable tissues were minced with trimming blades into 1-mm3 cubes in a 50-mm tissue culture dish (Falcon No. 3002) after sealing in EMEM supplemented with 20% heat-inactivated FCS, 100 µg/ml of sodium piperacillin, 5 µg/ml of gentamicin, and 5 µg/ml of amphotercin B. After preparation of a single cell suspension by mechanical pipetting in 20% EMEM containing 5 µg/ml of gentamicin, 1 × 107 cancer cells were seeded in each of two 100-mm tissue culture dishes (Falcon No. 3003) with 10 ml of medium preincubated at 37°C in a humidified 5% CO2/95% air atmosphere. Part of the medium was replaced by fresh medium every 2 or 3 days. On the 36th day after initiation of the culture, the first passage was performed. The trypsinized cells were placed into a 100-mm tissue culture dish containing 10% EMEM with a 1:4 split ratio, and the remaining cells were frozen at each passage.

Heterotransplantation. The cell suspension containing 1.6 × 107 cells in 1 ml of 10% EMEM was transplanted into the subcutis of BALB/c-nu/nu mice (4-week-old females; Kurea Co., Ltd., Tokyo, Japan). When the tumor reached about 1 cm in diameter, it was excised and examined histologically.

Cytological Studies. For light microscopy, KSE-1 cells were cultured on double-chamber Lab-Tek slides (Catalogue No. 4802; Miles Laboratories). The cells were then stained with hematoxylin and eosin.

For transmission electron microscopy, a single cell suspension of untreated cells, heterotransplanted tissue, and a biopsy specimen of the lymph node tumor were fixed with 2% glutaraldehyde (TAAB Laboratories Equipment Ltd.) in 0.1 M cacodylic acid buffer solution and postfixed with 1% osmium tetroxide in the same buffer solution at 4°C for 2 h. Materials were dehydrated in a graded series of ethanol (50-100%) and then embedded in Epon 812 (TAAB Laboratories Equipment Ltd.). Ultrathin sections were prepared with a JEOL 100CX electron microscope, after double staining with uranyl acetate and lead tannate.

Immunohistochemical Staining for Detection of Keratin. The avidin-biotin complex method (9) was used to detect keratin and hence confirm the squamous cell origin of the KSE-1 line. The KSE-1 cells were grown on double-chamber Lab-Tek slides, rinsed with PBS, and fixed in 100% ethanol for 10 min. The 5-µm sections of esophageal tissues were treated with 0.3% H2O2 in methanol for 30 min. The cultured cells and the sections of esophageal tissues were incubated with 3% normal swine serum for 30 min and then with rabbit anti-keratin serum (Dakoimmunoglobulins, Ltd., Denmark). 1/400, for 30 min at room temperature. The preparations were incubated with biotin-labeled secondary antibody for 30 min and then with avidin-biotin-peroxidase complex for 30 min (Vectastain ABC Kit). Finally, they were incubated in 0.01% hydrogen peroxide and 0.05% diaminobenzidine tetrahydrochloride in 0.05 M Tris buffer, pH 7.2, for 5 min and counterstained with 1% methyl green.

Chromosome Analysis. For confirmation of the human origin of the KSE-1 cells, chromosome analysis was done, using a modification of...
the G-banding technique (10). The cultured cells at the 50th passage were treated with Colcemid at a final concentration of 0.2 \( \mu \)g/ml for 7 h before the harvest, after which they were dispersed with 0.25% trypsin solution and washed in PBS. The washed cells were resuspended in a hypotonic solution containing 0.075 M KCl and were incubated for 10 min. After centrifugation, the pellet was fixed three times with the fixation fluid (methanol/acetic acid, 3/1) and the thus prepared cells were placed on clean slides and then were flame-dried. The chosen preparation was treated with 0.0125% trypsin solution in isotonic saline for 10–15 s at room temperature and was then stained for 10 min with Giemsa solution. Histograms of chromosome distribution and the karyotypic features were investigated by analyzing 90 metaphases.

Examination of Epstein-Barr Virus. Epstein-Barr virus antigen in the KSE-1 cell line was examined by anti-complement immunofluorescence antibody (Reedman and Klein) (11), and EBV DNA was examined by in situ hybridization with EBV Bio-Probe\textsuperscript{TM} Sequences (ENZO Biochemical Co., Ltd.) (12).

Detection of Mycoplasma. For detection of Mycoplasma in the KSE-1 cell line, a Hoechst Stain Kit (Catalogue No. 30-100-00) was used.

Cell Growth Responsive to Estradiol and Testosterone. The cell growth response to estradiol and testosterone was evaluated by measuring the cell number and thymidine incorporation. KSE-1 cells at the logarithmic growth phase were seeded into a 50-mm tissue culture dish at a density of 1 \( \times \) 10\(^5\) cells/dish. After incubation at 37°C in 5% CO\(_2\)/95% air atmosphere for 48 h, the cells were washed five times with serum-free medium and 5 ml of fresh EMEM containing 10% FCS/DCC were added, with or without various concentrations of estradiol and DHT.

The cell number was counted daily, after removal from the plates with 0.25% trypsin solution. The percentage of cell growth was determined after hormone treatment for 48 h.

The effect of hormones on \[^{3}H\]dThd incorporation was determined after hormone treatment for 24 h. \[^{3}H\]dThd (specific activity, 25 Ci/mmole) was added to each culture dish at a concentration of 1 \( \mu \)Ci/ml and the preparation incubated for 2 h at 37°C. The cells were washed eight times with PBS and then incubated at 37°C for 3 min with 1 ml 0.05% trypsin solution. The trypsinized cells were removed from the plates by repeated pipeting and were sonically disrupted and treated with trichloroacetic acid at 3000 \( \times \) g for 20 min. The pellets were dissolved in 0.3 N KOH and maintained at 37°C for 20 h. Using the scintillator (ACS-II, Amersham), the radioactivity was determined.

Hormone Receptor Assay. Approximately 1 \( \times \) 10\(^6\) trypsinized KSE-1 cells were sonically disrupted in 2 ml of TESH-glycerol buffer at 4°C, followed by centrifugation at 105,000 \( \times \) g for 60 min at 4°C. The supernatant served as the hormone receptor fraction.

Estrogen receptor assay was performed by a modification of the method of Tominaga et al. (13), in which the incubation mixture for the total binding study was composed of 50 \( \mu \)l of TESH-glycerol buffer containing various concentrations of 16a-\(^{125}\)I-17\(\beta\)-estradiol (7.5, 15.0, 30.0, 60.0, and 120.0 fmol/25 \( \mu \)l) and 50 \( \mu \)l of cytosol. The incubation mixture for the nonspecific binding study was composed of 50 \( \mu \)l of 2 \( \mu \)M diethylstilbestrol containing 16a-\(^{125}\)I-17\(\beta\)-estradiol and 50 \( \mu \)l of...
cytosol. After incubation for 18 h at 4°C, 0.5 ml of DCC was added and the preparation was further incubated for 30 min at 4°C, followed by centrifugation at 3000 x g for 20 min. The supernatant was directly assayed for 125I radioactivity, using an Aloka Auto-Gamma counter.

Testosterone receptor assay was done by the method of Shimazaki et al. (14), with some modification. The cytosol was incubated in 500 µl of TESH-glycerol buffer containing various concentrations of [1,2,6,7-3H]DHT and 10 mM Na2MoO4 at 4°C for 20 h. After incubation, 0.5 ml of TESH-glycerol buffer containing DCC was added and the preparation centrifuged. Radioactivity of the supernatant was determined in 5 ml of toluene (1 liter)/PPO (4 g)/POPOP (0.1 g) in a liquid scintillation counter. In parallel incubations, a 1000-fold molar excess of unlabeled ligand was added. Nonspecific binding not displaced under these conditions was subtracted.

The dissociation constant (Ka) and binding capacity were determined by Scatchard analysis (15). In the above method, the Ka on the high affinity of estrogen receptor was 10^{-11}–10^{-10} M.

RESULTS

Primary Culture and Establishment of the KSE-1 Cell Line. The epithelial-like cells grew gradually, forming a few clusters within 5 days of culture. Fibroblasts originating from the residual connective tissue grew slowly. The first successful subculture was accomplished on the 36th day and the second one was accomplished 10 days later. Thereafter, the cells were successively transferred at a split ratio of 1/4 every 7–10 days. With every subculture, the fibroblasts gradually decreased in number and in the 20th subculture, were absent. This KSE-1 subcultured cell line has gone through over 80 passages (July 1986) at 17 months after initiation.

Heterotransplantation. At 14 days after inoculation, all the heterotransplantations into the nude mice led to the formation of a tumor with an average diameter of 5.5 mm. At approximately 30 days, the tumors had reached an average diameter of 1 cm and then grew slowly for the next 38 weeks until all the mice died of tumor.

Morphology. Biopsy specimen of the esophageal tumor obtained at endoscopy before the initial surgery on our patient showed proliferation of spindle-shaped cancer cells arranged in a nest formation with focal keratinization, indicative of poorly differentiated squamous cell carcinoma (Fig. 1A). Light microscopic studies of lymph node tumor (Fig. 1B), KSE-1 cells transplanted into nude mice (Fig. 1C), and cultured cells on double-chamber Lab-Tek slides (Fig. 1D) revealed anaplastic spindle-shaped cell proliferation, with no differentiation.

Immunohistochemical studies using the avidin-biotin complex method revealed that lymph node tumor (Fig. 2A), KSE-1 cells transplanted into the nude mice (Fig. 2B) and the cultured cells (Fig. 2C) were all positive for keratin, thereby suggesting a squamous cell origin.

Transmission electron microscopy of the biopsy specimen of the lymph node revealed that these cells possessed a number of tonofilaments, lysosomes, ribosomes, and a few mitochondria (Fig. 3). The heterotransplanted KSE-1 cells also exhibited bundles of tonofilaments within the cytoplasm (Fig. 4). The cultured KSE-1 cells had an abundant Golgi apparatus, mitochondria, rough endoplasmic reticuli, free and membrane-bound ribosomes, and inclusions (16) as well as microvilli-like projections on the cell surface (Fig. 5). Some of the inclusions were electron dense and others were distinctly lamellar-like.

Chromosome Analysis. Chromosomes varied within a comparatively narrow hyperdiploid range from 61 to 72, with a modal number for the distribution of 67. As shown in Fig. 6, the clonal abnormalities, including nollisomy of chromosomes 4, 5, 6, and 14; monosomy of the X chromosome; 9p–, 12q+, and three distinct unidentified marker chromosomes were observed in all 90 cells. The KSE-1 line was confirmed to be of human origin from the banding pattern and the above-mentioned karyotypic features.

Examination of EBV and Mycoplasma. EBV antigen and EBV DNA in the KSE-1 line was not detected and the Mycoplasma test was negative.

Effects of Hormones on the Growth of KSE-1 Cells. Fig. 7 shows that the doubling time of KSE-1 cells cultured in 10% EMEM was approximately 19.5 h, as calculated by the growth rate at the logarithmic phase. This cell line lacks the density-dependent inhibition of cell growth; hence, there is a piling up...
phenomenon over approximately $2 \times 10^6$ cells/dish. On the 2nd day after treatment with various media, the cell number was $1.6 \times 10^6$ with 10% FCS, $7.8 \times 10^6$ with 10% FCS/DCC, $3.5 \times 10^5$ with FCS/DCC plus $10^{-9}$ mg/ml estradiol, and $1.2 \times 10^6$ with 10% FCS/DCC plus $10^{-7}$ mg/ml DHT, respectively. Growth rate of the cells was highest on medium not treated with DCC. The growth rate after treatment with FCS/DCC was higher than that with FCS/DCC plus estradiol and lower than that with FCS/DCC plus DHT. There were statistical differences.

Cell Growth Responsive to Estradiol and Testosterone. Fig. 8 shows the percentage of cell growth after treatment with various doses of estradiol and of DHT relative to cell growth after incubation in control medium. The cell number in case of treatment with estradiol at concentrations between $10^{-14}$ and $10^{-8}$ mg/ml fell in a dose-dependent manner (Fig. 8A), becoming 45% of the control value at an estradiol concentration of $10^{-9}$ mg/ml. The percentage of cell growth in case of treatment with DHT at concentrations between $10^{-13}$ and $10^{-6}$ mg/ml exceeded that of the control value (Fig. 8B) and again was found to be dose dependent, becoming 145% at a DHT concentration of $10^{-7}$ mg/ml.

Fig. 9 shows the percentage of the $[^{3}\text{H}]d\text{Thd}$ incorporation by cells treated with various doses of estradiol or DHT relative to the $[^{3}\text{H}]d\text{Thd}$ incorporation by cells incubated in control medium. $[^{3}\text{H}]d\text{Thd}$ incorporation in cells treated with estradiol was consistently lower than that in the absence of estradiol treatment (Fig. 9A). The percentage of the $[^{3}\text{H}]d\text{Thd}$ incorporation by the cells treated with $10^{-9}$ mg/ml estradiol was reduced to approximately 76% of the control value. $[^{3}\text{H}]d\text{Thd}$ incorporation by cells treated with DHT at concentrations between $10^{-13}$ and $10^{-6}$ mg/ml exceeded that in the untreated controls (Fig. 9B). The percentage of the $[^{3}\text{H}]d\text{Thd}$ incorporation in cells treated with $10^{-7}$ mg/ml DHT was 120% of the control value.

Scatchard analysis (Fig. 10) revealed that KSE-1 cells had a $K_d$ of $0.35 \times 10^{-10}$ M and a binding content of 4.2 fmoles/mg protein for the estradiol receptor, and a $K_d$ of $0.69 \times 10^{-11}$ M and a binding content of 2.2 fmoles/mg protein for the DHT receptor. The specific bindings of both estradiol and DHT yielded a single straight line, indicating that a single class of receptor sites was present, in the range of concentrations studied. Saturation was reached when the estradiol concentration was $2.4 \times 10^{-15}$ M and the DHT concentration was $2.9 \times 10^{-11}$ M. Both receptors were of high affinity, of low capacity, and saturable.
DISCUSSION

We established a new cell line (KSE-1) from squamous cell carcinoma derived from a human esophageal cancer. Although histological study of the metastasized axillary lymph node tumor, cultured cells, and transplanted tumor cells revealed no evidence of differentiation, immunohistochemical staining for detection of keratin and transmission electron microscopic studies revealed that these cells were of squamous cell origin. Thus, we concluded that these cells were derived from metastatic esophageal squamous cell carcinoma. We confirmed that these cells were of human origin by doing a chromosome analysis.

It has been reported that cell proliferation in tissues derived from breast cancer cells (17), prostatic cell carcinoma (18), and other human solid carcinomas (19) is regulated by sex hormones. However, there is little documentation on the influence of sex hormones on squamous cell carcinoma (6) and no reports on the response to such hormones on human squamous cell carcinoma derived from esophageal cancer.

The KSE-1 cell line has a binding content of 4.2 fmol/mg protein for the estrogen receptor and 2.2 fmol/mg protein for
the testosterone receptor in the cytosol. Growth of this cell line is moderately responsive to both estrogen and testosterone at these concentrations up to $10^{-12}$ mg/ml and including a confluent layer of cells. Beyond this concentration, response to hormones is masked by other factors including nutrition, O$_2$ diffusion, etc. Furthermore, the absolute response to estrogen is higher than in human mammary carcinoma cells and lower than in normal human mammary epithelial cells, as reported by Hankim (20). Realizing the negative nature of this response for estrogen (20, 21), it is tempting to consider that estrogen inhibits growth of the KSE-1 line in a dose-dependent manner up to a maximum concentration of $10^{-12}$ mg/ml estradiol.

Beyond this, cell growth, although still inhibited, becomes independent of a further increase in estrogen concentration. We believe that this probably reflects hormone receptor saturation at the cellular level. Likewise, testosterone was found to enhance cell growth up to a maximum concentration of $10^{-8}$ mg/ml DHT at which point saturation occurred and no further enhancement was noted for higher doses.

The DHT receptor content of our KSE-1 line, 2.2 fmol/mg protein, is of the same magnitude as the estrogen receptor content noted by McGuire (22) for breast carcinoma, i.e., under 3.0 fmol/mg. Since their study indicated a response to endocrine therapy of approximately 9%, it is reasonable to assume that similar therapy would yield a comparable result when
comparative studies with low passage normal and tumor cells are currently under way with female mice.

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