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Estrogen Receptor Levels and Tumor Growth in a Series of Pituitary Clonal Cell Lines in Rats

Nariaki Fujimoto,[1] Hiromitsu Watanabe,[1] Akihiro Ito[1] and Kinji Inoue[2]

[1]*Department of Cancer Research, Research Institute for Nuclear Medicine and Biology, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734 and* [2]*Department of Morphology, Institute of Endocrinology, Gunma University, Showa-machi 3-39-15, Maebashi 371*

Four kinds of *in vitro* clonal pituitary tumor cell lines named MtT/Se, MtT/SM, MtT/S and MtT/E, each of which shows different sensitivity to estrogen on proliferation, were inoculated into fat pad of ovariectomized rats and estrogen-loaded ovariectomized rats at 10^5 and 10^6 cells/site. They formed tumor with average latency ranging from 30 to 71 in ovariectomized rats and 13 to 63 days in estrogenized rats inoculated with 10^6 cells. MtT/Se was highly sensitive to estrogen for growth, and MtT/SM also grew well in estrogenized rats. With MtT/S and MtT/E, there was no significant shortening of average tumor latency in estrogenized rats. *In vivo*, the cytosolic estrogen receptor (ER) levels of MtT/Se, SM, S and E were measured to be 452 ± 66, 370 ± 115, 260 ± 16 and 83 ± 8 fmol/mg protein, respectively. *In vitro*, however, the lowest ER level was noted in MtT/Se. Histologically, all four tumors grown in rats were composed of homogeneous round cells, and MtT/Se contained particularly large nucleated cells. In MtT/E, the cells appeared to be changing into fibromatous cells. Three cell lines except MtT/E maintained the function of hormonal secretion *in vivo* as well as *in vitro*. Serum GH level was increased in rats with MtT/Se and MtT/S. Increased levels of both prolactin and growth hormone were measured in sera of rats with MtT/SM. Increases of hormones as well as tumor sizes were promoted by the estrogen.

Key words: Pituitary tumor — Estrogen-dependent growth — Estrogen receptor

It is known that long-term stimulation with estrogen leads to pituitary tumors in rats. These pituitary tumors or clonally cultured cells have been used to study the factors involved in growth and hormone production.[1,2] Estrogen-induced pituitary tumor grows well in the presence of estrogen in the early passages. Upon subsequent passages, however, they give rise to "autonomous variants," and they become transplantable even in ovariectomized rats.[3,4] In estrogen-dependent growth of pituitary and mammary tumors, binding of the hormone to the estrogen receptor (ER) is considered to be the first step.[5–9] As has been established in human breast cancers, hormonal therapy was quite effective to diminish the tumor size if the tumors contained a significant amount of ER.[10,11] In some models, however, disappearance of ER was not a prerequisite for the development of autonomous variants independent of estrogen.[12–14] Autonomous growth might be related to post-ER pathways.

We have established a series of pituitary clonal cell lines differing in growth response to estrogen *in vitro*.[15] In the present study, we inoculated these cells into ovariectomized rats and estrogen-loaded ovariectomized rats and observed their tumorigenic potentials in relation to ER levels. We further measured serum prolactin (PRL) and growth hormone (GH) levels in rats when the tumors became palpable.

Abbreviations: GH, growth hormone; PRL, prolactin; MtT, mammotropic tumor; ER, estrogen receptor; HS, horse serum; FBS, fetal bovine serum; E_2, 17β-estradiol.

MATERIALS AND METHODS

***In vitro* cell lines** Four cell lines, MtT/Se, MtT/SM, MtT/S and MtT/E were cultured in DEM/F12 mixed medium (Sigma Chemicals D-8900) with 10% horse serum (HS, Gibco) and 2.5% fetal bovine serum (FBS, Gibco). Details of the cell culture were described previously.[15]

Inoculation of cells F344 female rats, 28 days old, were obtained from Charles River Japan Co., Kanagawa. Animals were maintained according to the "Guide for the Care and Use of Laboratory Animals" by Hiroshima University. All animals were surgically ovariectomized one week before tumor cell inoculation. When cultured cells reached a density of about 5×10^7 per 10 cm dish, the cells were harvested with 0.02% trypsin and counted with a hemocytometer. Cell suspension was mixed with an equal volume of 50% brain homogenate (brains were obtained from syngeneic adult rats and homogenized in DEM/F12 mixed medium as described previously[16]). The mixture was inoculated into two different sites per rat at a volume of 0.06 ml/site into subcutaneous fat pads. The grafted sites were observed twice a week. Tumor formation was evaluated by palpation of the grafted sites. After the tumor had reached 1–2 cm in

diameter, the animals were killed under ether anesthesia. Blood samples were collected from the abdominal artery and separated sera were stored at −20°C until required for assays. The tumors were fixed in 10% neutral formalin solution and embedded in paraffin. Deparaffinized sections of 3 μm in thickness were stained with hematoxylin and eosin (HE). For ER assay, the tumor tissues were frozen with liquid nitrogen and stored at −70°C.

PRL and GH levels Radioimmunoassays for PRL and GH were carried out with NIDDK (National Institute of Diabetes and Digestive and Kidney Diseases) reagents, in accordance with the recommended methods. Iodination was performed by the lactoperoxidase method ($Na^{125}I$, Amersham). The second antibodies, anti-rabbit immunoglobulin for PRL assay and anti-monkey immunoglobulin for GH assay, were gifts from the Institute of Endocrinology, Gunma University.

ER assay for cells and tumor tissues All steps were conducted at 0–4°C. The cell pellet or the tumor tissues were homogenized in TEDMG buffer (10 m*M* Tris-HCl, 1 m*M* disodium EDTA, 1 m*M* dithiothreitol, 10 m*M* sodium molybdate and 10% (v/v) glycerol, pH 7.4) with a Physcotron (Niti-On, Tokyo). The homogenates were centrifuged at 800*g* for 10 min to separate the nuclear pellets. The supernatants were further centrifuged at 105,000*g* for 50 min to obtain cytosol. The nuclear pellets were washed in TEDMG buffer and resuspended in TEDMG buffer containing 0.5 *M* KCl (pH 7.4). After incubation in ice bath for 60 min, they were centrifuged at 105,000*g* for 50 min to obtain nuclear salt extracts. Protein concentrations in cytosols were measured by using Lowry's method and DNA concentrations in nuclear suspensions were determined by using a dye, bisbenzimidazole.[17]

Each cytosol and nuclear salt extract was incubated with a selected concentration (0.05–5 n*M*) of 17β-2,4,6,7-^{3}H-estradiol (New England Nuclear) or ^{3}H-estradiol plus a 100-fold molar excess of unlabeled estradiol (5–500 n*M*) for 40 min at 30°C (0.2 ml in final volume). One volume of 60% (v/v) hydroxylapatite (Bio-Gel HAP, Bio-Rad, Richmond, CA) suspension was added to each tube, then it was incubated for 10 min at 4°C with occasional vortexing. Cold TEDMG buffer (2 ml) added to each tube of the incubated suspension and the tubes were centrifuged at 800*g* for 10 min. The pellets were washed twice with 2 ml of TEDMG buffer and ^{3}H-estradiol was extracted with 1 ml of ethanol. The ethanol solutions were transferred into counting vials and the radioactivity was counted.

RESULTS

Tumor incidence and latency of the four clonal cell lines in rats The results are summarized in Table I. MtT/Se showed high sensitivity to estrogen in forming tumors in rats. After inoculation of 10^5 cells, MtT/Se started to form tumors on day 22 in estrogenized rats, while the first tumor was long delayed in the ovariectomized counterparts. After inoculation of 10^6 cells, the average tumor latency was greatly shortened to 13 days in estrogenized rats and 30 days in ovariectomized rats. Inoculation of MtT/SM (10^5 cells) into estrogenized rats did not form any tumor before day 162, but two tumors within ten grafted sites (20%) were observed in ovariectomized rats on day 162. When 10^6 MtT/SM cells were grafted, however, tumors formed rapidly with high incidence in estrogenized rats and in ovariectomized rats as well. The first tumor was observed on day 20 in an estrogenized rat

Table I. Incidence and Latency of Tumors Formed by Individual Cell Lines Grafted into Ovariectomized or Estrogenized Rats

Type	No. of cells inoculated	Overiectomized female		Estrogenized female	
		Incidence	Latency (days)	Incidence	Latency (days)
Se	1×10^5	1/10	>162	10/10	22–44 (22)
	1×10^6	11/12	30–79 (30)[a]	12/12	13–30 (13)
SM	1×10^5	2/10	≥162	0/10	>162
	1×10^6	10/10	51–100 (63)	11/12	20–63 (30)
S	1×10^5	10/10	83–108 (94)	10/10	49–80 (65)
	1×10^6	11/12	53–91 (71)	11/12	42–70 (63)
E	1×10^5	12/12	63–102 (88)	9/12	51–66 (63)
	1×10^6	11/12	30–40 (40)	11/12	20–40 (30)

a) Values in parentheses show the latency for 50% incidence.

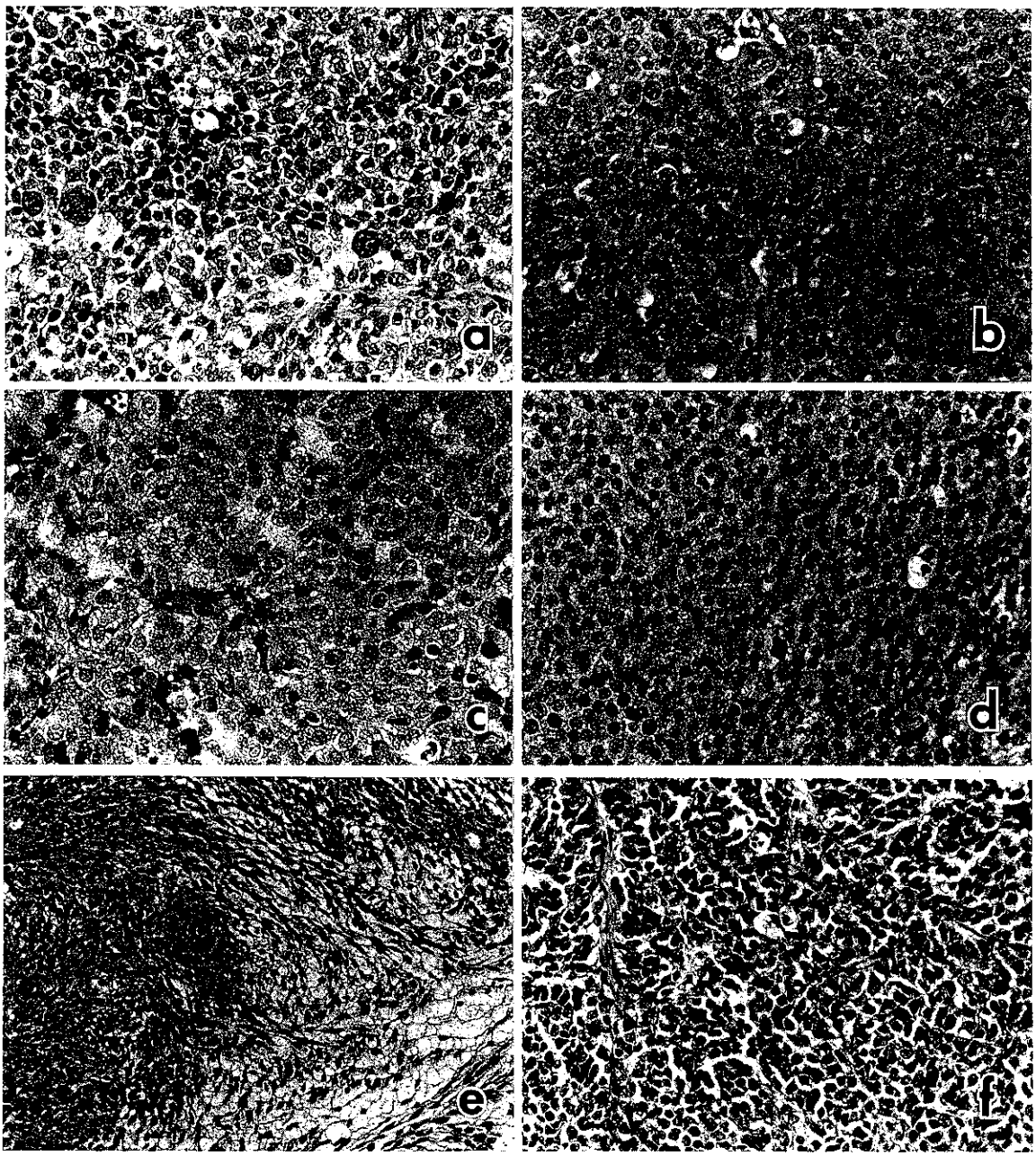

Fig. 1. Microscopic appearance (HE) of MtT/F84 and its four clonal cell lines grafted in rats. a) MtT/Se is chracterized by appearance of numerous large nucleated cells and round tumor cells (×700). b) MtT/S, c) MtT/SM and d) MtT/E showed homogeneous round cells with scanty cytoplasm and occasionally mitotic cells (×700). e) MtT/E cell line has a fibromatous appearance with partly myxomatous changes (×350). f) Original MtT/F84 (×700).

and on day 51 in ovariectomized rats. After inoculation of 10^5 MtT/S cells, tumor formation began on day 49 in estrogenized rats and day 83 in ovariectomized rats. Average latency was not much shortened by inoculation of 10^6 cells. MtT/E showed only slight shortening of latency in estrogenized rats compared to that in ovariectomized rats after inoculation of either 10^5 or 10^6 cells.

Histological findings As shown in Fig. 1, MtT/Se, MtT/SM and MtT/S were typical pituitary adenomas like the original MtT/F84. In MtT/Se, particularly large nuclei

Table II. *In vitro* Characters of Four Cell Types[a, b]

Type	Hormonal activities[c]		Cytosolic ER		Doubling time[d] (h)	Estrogen sensitivity on proliferation[d]
	GH (ng/ml/10^6)	PRL	Specific binding (fmol/mg protein)	K_d (n*M*)		
Se	19±2	0	27±3.5	0.37±0.07	24	++
SM	10,200±250	257±16	90±12.2**	0.34±0.06	50	+
S	15,700±690	0	82±5.5**	0.46±0.03	43	±
E	0	0	55±2.5**	0.46±0.03	55	−

a) Data are presented as mean±SE (N=5–6).
b) The culture medium (described in "Materials and Methods") contains endogenous estrogen derived from horse and fetal bovine sera.
c) GH and PRL levels were measured at day 4 after cultivation of 10^6 cells.
d) Doubling time and estrogen sensitivity on proliferation were examined as reported earlier.[15]
** Significantly different from Se ($P<0.01$).

Table III. Estrogen Receptor Levels in Tumors Grown in Rats from Four Cell Lines by Inoculation of 1×10^6 Cells[a]

Type	E_2	cER		nER	
		Specific binding (fmol/mg protein)	K_d (n*M*)	Specific binding (fmol/mg DNA)	K_d (n*M*)
Se	−	452±66	0.83±0.09	267±47	0.17±0.01
SM	−	370±115	0.99±0.21	272±51	0.23±0.05
S	−	260±16*	0.76±0.05	164±21**	0.20±0.03
E	−	83±8**	0.60±0.08	23±9**	0.40±0.06
Se	+	476±45	0.74±0.12	819±156	0.20±0.03
SM	+	185±10**	0.82±0.09	374±117	0.13±0.02
S	+	104±22**	0.41±0.06	226±32**	0.17±0.02
E	+	156±33**	0.64±0.07	123±27**	0.22±0.02

a) Data are presented as mean±SE (N=3–6).
*, ** Significantly different from Se: $P<0.05$ (*) or $P<0.01$ (**).

were observed (Fig. 1a), while in MtT/E, fibroblastic changes of tumor tissue were predominant (Fig. 1e).

ER levels in cultured cells and in grafted tumors *In vitro* hormonal activities, ER levels and cell growth with estrogen in the four clonal cell lines are shown in Table II. MtT/Se showed a rather low level of ER and the other three cell lines showed relatively high levels of ER. No significant difference was noted in dissociation constants (K_d) among the four cell types.

In grafted tumors, cytosolic ER levels were generally higher than those *in vitro* (Tables II and III). Among the four cell lines grown in ovariectomized rats with or without estrogen, cytosolic ER levels were much higher in MtT/Se compared to those in MtT/S or MtT/E. Comparing the three tumors except MtT/E between ovariectomized and estrogenized rats, nuclear ERs showed relatively high levels and cytosolic ERs were relatively low in estrogenized rats.

Table IV. Serum GH and PRL Levels in Tumor-bearing Rats[a]

Type	E_2	Serum GH (ng/ml)	Serum PRL (ng/ml)	Tumor size (mm)
Se	−	16±4.3	9±2.1	7±1.2
	+	125±12**	10±1.9	16±1.5
SM	−	2,760±1,650	39±29	9±1.2
	+	4,410±1,340	755±269*	19±3.2
S	−	637±130	10±2.5	10±1.8
	+	2,010±507*	52±22	12±1.3
E	−	5±0.8	6±0.7	14±1.0
	+	12±2.4*	16±3.6*	14±3.0

a) Data are presented as mean±SE (N=5–6), and tumor size is presented as the average of two perpendicular diameters at the time of death.
**, * Significantly different from E_2- in each cell type: $P<0.05$ (*) or $P<0.01$ (**).

Hormonal activities The four clonal cell lines showed different hormonal activities. GH and PRL concentrations in the medium were measured at day 4 after cultivation of 10^6 cells (Table II). *In vitro,* MtT/Se produced a small amount of GH. MtT/SM and S produced large amounts of GH and MtT/SM produced PRL as well. MtT/E did not produce GH or PRL. When grafted into estrogen-treated rats, each tumor type maintained almost the same hormonal activities as *in vitro* (Table IV). It is also quite clear that estrogen treatments significantly increased serum GH level both in MtT/Se- and MtT/S-bearing rats and also increased serum PRL level in MtT/SM.

DISCUSSION

The present study has demonstrated that all four clonal cell lines established recently[15] have potency to form tumors. The spectra of hormone production in these tumors were similar to those *in vitro.* We found some discrepancy of ER levels between *in vivo* and *in vitro.* In MtT/Se, which showed the highest estrogen sensitivity of growth among the four cell lines, ER level was the highest *in vivo,* but the lowest *in vitro.* Although we have no evidence, it is possible that the greater amount of ER in MtT/Se cells may be related to the higher growth rate *in vivo,* and high-ER cell clones may be selected during tumor formation.

Growth of MtT/F84, from which the four clonal cell lines were obtained, was well correlated to the administered doses of estrogen.[16, 18] Estrogen-induced growth stimulation in pituitary and mammary tumors has been postulated to occur through ER.[5–9] The presence of measurable ER, however, does not always mean that the tumor is estrogen-dependent. In studies on estrogen-dependent human mammary carcinoma cell line MCF-7, from which tamoxifen-resistant variants were isolated, no significant difference was detected in ER levels between variants and the original line.[12–14]

The nuclear ER level of every cell type in Table III was always higher in the tumor grown in estrogen-loaded rats compared to those without estrogen. These findings suggest that translocation of ER from cytoplasm to the nuclear region, following the classical "two-step" hypothesis,[19] operates in all cell types.

Every cell type has a potency to produce different pituitary hormones. *In vitro,* MtT/Se and S secrete only GH and MtT/SM secretes both PRL and GH. When tumors were grown in rats, each cell line maintained almost the same properties of hormonal activities as *in vitro* (Table IV). In MtT/SM-bearing rats, interestingly, administration of estrogen increased only serum PRL level, but not GH. In MtT/Se- and MtT/S-bearing rats, however, significantly high GH levels were induced by the administration of estrogen. In MtT/W15 tumor, diethylstilbestrol treatment stimulated GH synthesis but not PRL,[1, 2, 20] whereas estrogens stimulate PRL and decrease GH synthesis in normal pituitary.[21, 22] In this respect, MtT/SM behaved like normal pituitary.

We are currently interested in the estrogen-related regulatory mechanism of tumor cell growth, and as the next step in our research, we intend to study these estrogen-sensitive and hormone-producing pituitary cell lines in detail.

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