Jpn. J. Cancer Res. 82, 815–819, July 1991

Stimulatory Effects of Retinoic Acid on Tumor Growth and Serum Insulin-like Growth Factor-1 in Rats Bearing Estrogen-responsive Pituitary Tumor MtT/Se

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MtT/Se is one of 4 cell lines derived from an estrogen-dependent pituitary tumor, MtT/F84. The main difference between these tumor types is that MtT/F84 secretes both growth hormone (GH) and prolactin (PRL) whereas MtT/Se secretes only GH. MtT/Se grew slowly in ovariectomized (ovex) rats, but tumor growth was much faster in estrogen-treated ovex rats. Effects of dietary retinoic acid (RA) on tumor growth, serum GH and insulin-like growth factor-1 (IGF-1) levels were examined in ovex rats. Latency of tumor growth was shortened, and tumor take and weight were promoted by all-*trans* RA both in the presence and absence of exogenous estrogen. Serum GH and IGF-1 levels became increased in tumor-bearing rats whereas PRL levels remained unchanged. Serum IGF-1 levels exhibited a good correlation with tumor weights ($r=0.84$). Our results suggest a close relationship between increase of tumor weight and stimulation of serum IGF-1 level by RA in tumor-bearing rats.

Key words: Pituitary tumor — Retinoic acid — Tumor growth — Insulin-like growth factor-1 — Estrogen

All-*trans* retinoic acid and its natural and synthetic analogs have a significant protecting effect against neoplastic development in experimental animals. Retinoids have been shown to have anticarcinogenic activity in animal tumor models such as tumors of mammary gland,[1] urinary bladder,[2] pancreas,[3] skin,[4] lung[5] and oral cavity[6] and other tissues. RA[2] also promotes tumor growth, as has been observed in diethylnitrosoamine-induced liver tumor[7] and estrogen-dependent pituitary tumor.[8] RA also plays an important role in development,[9] cell differentiation[10] and induction of specific gene expression.[11] It also regulates GH gene expression in GH_3[12] and GH_1[13] cell lines. Furthermore, Morita *et al.*[14] demonstrated that it acts additively with tri-iodothyronine for the stimulation of GH gene expression and GH secretion in culture medium of GH_3 cells without any effect on PRL synthesis. Recently, Hirose *et al.*[15] reported that RA stimulates growth of estrogen-responsive transformed murine Leydig cells *in vitro*. They suggested that RA regulates growth of this cell line with the help of RAR rather than ER, which is also present.

MtT/Se is one of the cell lines derived from an E-dependent pituitary tumor, MtT/F84.[16] It has been demonstrated that growth of this cell line is E-dependent and the cells secrete GH, but not PRL, in culture media. In the previous report[8] we have shown that growth of the original tumor, MtT/84 which secretes both GH and PRL, is promoted by dietary RA in rats. In this report we deal with the effect of dietary RA on the growth of MtT/Se cell line and serum hormone levels *in vivo*.

MATERIALS AND METHODS

Grafting of tumor cells Young female rats, 4 weeks old, purchased from Charles River Co. (Tokyo), were ovariectomized, and grafting of tumor cells was done as described previously.[17] In this experiment, *in vitro*-cultured cells (about 10^5 cells/site) were grafted s.c. into 6 sites of the breast fat pads of each rat. After tumor cell grafting, rats were divided into the following groups containing 5 rats/group (i.e. 30 grafting sites/group in total): control, RA50, E and E+RA50 groups. Rats were implanted with 0.1 mg of E-pellets (17β-estradiol, Sigma Chemical Co., E-9000) on the back or/and given RA (all-*trans* retinoic acid, Sigma Chemical Co., R-2625)-enriched diet (50 mg/kg basal diet) on the same day according to the protocol described above. Ten days after grafting, inoculated sites were checked for tumor growth under mild ether anesthesia once every 3–4 days until autopsy. Rats were killed when tumors reached more than 1.0 cm in diameter. Tumors were stored at −80°C for receptor assay.

Preparation of tumor cytosol and nuclear extract ER levels were assayed within 3 months of tumor collection. Tissue was homogenized in freshly prepared TED buffer (10 m*M* Tris, 10 m*M* EDTA, 1 m*M* dithiothreitol, pH 7.4) at 4°C. The homogenate was centrifuged at 800*g* for

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[2] Abbreviations used: Ovex, ovariectomized; RA, retinoic acid; E, estrogen; ER, estrogen receptor; RA50, rats treated with retinoic acid at the dosage of 50 mg/kg basal diet; GH, growth hormone; PRL, prolactin; IGF-1, insulin-like growth factor-1; NRS, normal rat serum, RIA, radioimmunoassay; RAR, retinoic acid receptor.

10 min at 4°C to separate nuclear pellet and supernatant. The supernatant was centrifuged at 90,000*g* for 60 min to get cytosol for assay. The nuclear pellet was washed twice in TED buffer and suspended in KTED buffer (TED buffer containing 0.6 *M* KCl). The suspended pellet was sonicated for 10–12 s, incubated at 4°C for 60 min and centrifuged at 90,000*g* for 60 min to obtain nuclear salt extract.

ER assay The procedure was based on the reports of Bronzert *et al.*[18] and Ginsburg *et al.*[19] Aliquots of cytosol and nuclear extract were incubated with 1–100 nm [^{3}H]E (2,4,6,7[^{3}H]E, 89.9 Ci/mmol, NEN) in the presence or absence of 1,000-fold excess of non-radioactive E. After 30 min of incubation at 30°C, unbound E was absorbed by the dextran-coated charcoal (DCC) method and the supernatants were counted. Protein and DNA concentrations of cytosol and nuclear extract were measured by the methods of Lowry *et al.* and Labarca and Paigen,[20] respectively. Data were plotted according to Scatchard to calculate the maximum number of binding sites per mg protein or DNA.

GH and PRL RIA Hormone levels in serum were determined by the double antibody method. Rat GH and PRL and antibodies against GH and PRL were gifts from NIADDK (National Institute of Diabetes & Digestive Kidney Diseases, USA). Goat anti-monkey IgG and anti-rabbit IgG were gifts from Dr. Wakabayashi (Gunma University, Maebashi). Student's *t* test was used to determine the statistical significance of differences.

IGF-1 RIA Acid-ethanol extraction of serum was performed according to Daughaday *et al.*[21] Briefly, 0.8 ml of acid-ethanol mixture (87.5% ethanol, 12.5% *N* HCl) and 0.2 ml of serum were mixed thoroughly and kept for 30 min at room temperature. Tubes were centrifuged at 10,000 rpm for 5 min, and the supernatant was neutralized with 0.2 ml of 0.85 *M* Tris base to make it ready for RIA. IGF-1 RIA kit (Amersham, Tokyo) was used in this experiment. Briefly, the incubation mixture consisted of 100 μl of standard/unknown sample, 100 μl of anti-human IGF-1 rabbit antisera (1:4,000) and 100 μl of tracer ([3-^{125}I]iodotyrosyl IGF-1(Thr^{59}), 1,900 Ci/mmol). After 48 h incubation at 4°C, free IGF-1 was absorbed by the DCC method and the supernatants were counted in a gamma counter. Non-specific binding was measured in the presence of 500 ng of non-radioactive IGF-1.

RESULTS

Tumor growth Diet consumption by each rat, in basal and RA-enriched diet groups, was observed to be more or less the same, 8.0±0.2 g/day/100 g of body weight. No tumor was noted on the 20th day after grafting. On day 25, tumor takes were 0, 0, 7 and 30% in rats of the control, RA50, E and E+RA50 groups, respectively (Fig. 1). On day 34, tumor takes were noted as 0, 7, 27 and 77% in the control, RA50, E and E+RA50 groups,

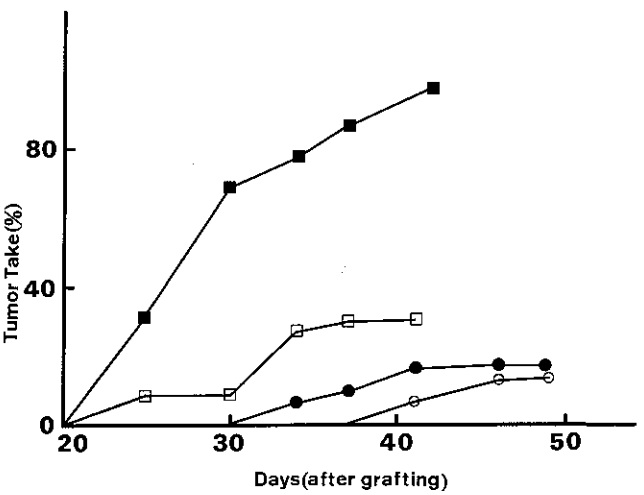

Fig. 1. Incidence of tumor take in rats of the control (○), RA50 (●), E (□) and E+RA50 (■) groups.

Table I. Effect of Retinoic Acid on Tumor Growth and ER Levels in Tumors Grown in Ovex Rats[a]

Treatment for tumor growth	Incidence of tumor (%)	Average tumor weight (g)	Average cytosolic ER[c] (fmol/mg prot)	Average nuclear ER (fmol/mg DNA)
Control	20	0.12±0.06	235±22	nd[d]
RA 50	20	0.28±0.21	280±35	nd
E	30	0.24±0.11	829±107	156±34
E+RA50	97	0.78±0.57[b]	917±72	109±15

a) Rats of control, RA50, E and E+RA50 groups were killed 68 and 42 days after cell grafting.
b) Difference between E and E+RA50 is significant ($P<0.01$).
c) Each datum of ER is the average of 3–4 different tumors.
d) Not determined.

Table II. Effect of Retinoic Acid on Serum GH, PRL and IGF-1 Levels in Ovex Rats Grafted with MtT/Se

Treatment	GH level (ng/ml)	PRL level (ng/ml)	IGF-1 (ng/ml)	Body weight[d] (g)
Control	4±1	20±11	125±48[b]	215±8
RA50	6±3	7±3	229±24	195±7
E	12±6	12±9	166±66	165±3
E+RA50	83±31[a]	18±14	259±32	162±6
NRS	6±2	26±4	159±2[c]	—

a) Value of E+RA50 is significantly different ($P<0.01$) from those of other groups.
b) Values of control vs. RA50 and E vs. E+RA50 are significantly different ($P<0.01$ and $P<0.05$, respectively).
c) Value was taken from a different experiment.
d) Measured when rats were killed at 68 and 42 days after cell grafting.

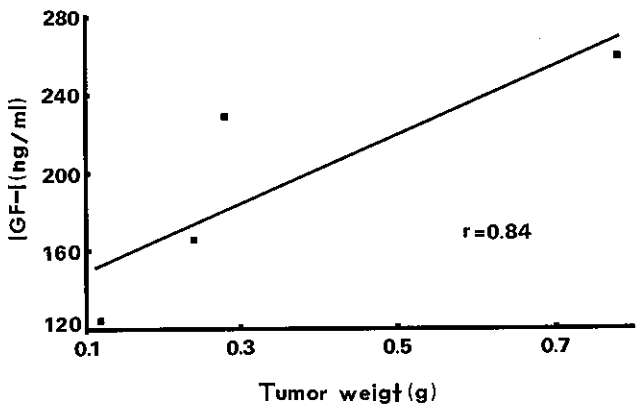

Fig. 2. Correlation between tumor weight and serum IGF-1 level in MtT/Se-bearing rats of the control, RA50, E and E+RA50 groups.

respectively. After 42 days, tumor take was 30 and 97% in the E and E+RA50 groups, whereas it was 20% in the control and RA50 groups even at 68 days after grafting. Tumor weight in the E+RA50 group was significantly higher than that of the other groups ($P<0.01$)(Table I).

ER levels Cytosolic ER levels were greatly increased in tumors of E- and E+RA50-treated rats compared with those of the control and RA50 groups. Nuclear ER levels were not affected in tumors of any group (Table I).

Serum GH, PRL and IGF-1 levels RA affected the growth of rats, as reflected by decreased body weights in RA-treated rats. Average GH levels were increased significantly from 12 to 83 and from 6 to 83 ng/ml (Table II) when ovex rats were treated with E+RA50. PRL levels were more or less the same in rats among different groups. IGF-1 levels were elevated in tumor-bearing rats when they were treated with RA50 both in the presence or absence of exogenous E ($P<0.01$). The elevated IGF-1 levels exhibited a good correlation ($r=0.84$) with tumor weights (Fig. 2).

DISCUSSION

It is clear from the present results that growth of MtT/Se in rats was promoted by RA as well as by E, as in the case of the original tumor, MtT/F84. This observation was further confirmed by a close relationship between E and RA for growth of this cell line *in vivo*. Recent studies revealed that ER and RAR genes belong to the same steroid hormone receptor gene superfamily[22] and they may have some common functions. We have also measured ER levels in tumors grown under various conditions. It was previously reported[8] that RA does not interfere with ER measurement. Although cytosolic receptor levels were elevated in the E and E+RA50 groups, nuclear ER levels remained unaffected even though tumor growth was promoted by RA (Table I). Nevertheless, measurement of both cytosolic and nuclear ER may be important since cytosolic receptor can be interpreted in terms of weak binding of ER to nuclear sites.

It was also reported that E and RA, which may be present in sera, do not interfere with GH measurement.[8] Serum GH increased only in E+RA50-treated ovex rats without any effect on body weight though the observation period may be too short for manifestation of the biological effect of increased GH level. Serum GH level may depend mainly on tumor growth, as has been observed in MtT/F84.[8] Serum PRL levels from different groups were more or less the same, being close to the value in NRS. These results also confirmed the *in vitro* characteristics of MtT/Se cell as a secretor of GH, but not PRL, into the medium. Serum IGF-1 level was elevated in the RA50 group and the highest value was noted in the E+RA50–treated group, as for serum GH. Rat pituitary tumors, either induced or transplanted, produce and secrete abundant GH into serum.[23,24] Morita *et al.*[12] and Bedo *et al.*[13] have shown that RA promoted synthesis and secretion of GH in culture media of GH_3 and GH_1 cell lines. Our results also confirmed that the serum GH level is increased in E+RA50-treated rats grafted with MtT/Se.

Increase of serum IGF-1 level in tumor-bearing rats might be due to stimulation of synthesis either in the tumor or in some other organ, e.g. liver. Although serum IGF-1 levels and tumor weights are well correlated ($r=0.84$), it is not clear from this study whether the increase of serum IGF-1 level is due to increased synthesis in the tumor or not. To resolve this point, expression of IGF-1 gene in tumor tissue is to be investigated. Although there

is a report of alteration of oncogene expression by RA,[25] our data lead us to consider two possible mechanisms to correlate E and/or RA treatment, tumor growth and increase of both serum GH and IGF-1 levels. Firstly, E may act on tumor cells through its receptor to promote tumor growth and to increase serum GH and IGF-1 levels, and RA may act synergistically with E. Secondarily, RA may act independently through its binding protein or receptor to modulate tumor growth and to increase serum GH and IGF-1 levels.

It will be interesting to study whether GH plays any role through its mediator, insulin-like growth factor-1, in the growth of the tumor. To our knowledge, this is the first model in which RA may stimulate IGF-1 synthesis *in vivo*. If RA directly regulates IGF-1 synthesis in the tumor, this model may be useful for investigation of the effects of RA on gene expression.

ACKNOWLEDGMENTS

The authors thank Dr. K. Inoue (Gunma University) for providing MtT/Se. This work was supported in part by a Grant-in-Aid from the Ministry of Health and Welfare of Japan.

(Received March 15, 1991/Accepted May 2, 1991)

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