

Growth-promoting Effect of Retinoic Acid in Transplantable Pituitary Tumor of Rat

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MtT/F84 grew well in Fischer rats (F344), but tumor growth was promoted in hyperestrogenized rats. Effects of dietary retinoic acid (RA) on tumor growth, estrogen receptor (ER) and serum growth hormone (GH) level were examined. Tumor latency became shortened, and tumor take and weight were promoted by all-*trans* RA at dosages of 50 and 200 mg/kg basal diet, but not dose-dependently. ER level was elevated in tumor of RA-treated rats, whereas the retinoic acid-binding protein level remained unchanged. RA also elevated incorporation of 5-bromo-2'-deoxyuridine, a thymidine analogue, into DNA of tumor cells. Average serum GH level was increased in tumor-bearing rats treated with RA and was well correlated with tumor weight. RA may directly affect ER level and enhance estrogenic action, resulting in promotion of tumor growth, or it may act independently for tumor growth and elevation of serum GH level.

Key words: Estrogen-dependent pituitary tumor — Retinoic acid — Tumor growth — Estrogen receptor — Serum growth hormone

Retinoic acid plays an important role in development,¹⁾ cell differentiation²⁾ and induction of specific gene expression.³⁻⁵⁾ It also regulates growth hormone gene expression in GH₃⁶⁾ and GH₁⁷⁾ cell lines. RA² has been found to play a role in the prevention of epithelial cell carcinogenesis.⁸⁾ Gene expression of malignant cell lines as well as transcriptional activity of several genes in normal cells^{4,9)} are reported to be influenced by RA. It was reported that vitamin-A deficient diet reduces nuclear T₃-receptor in rat liver.¹⁰⁾ Estrogen, on the other hand, has a tumorigenic effect on pituitary, mammary tissue and uterus. Numerous possible mechanisms of action on cellular growth in mammary tumor by estrogen have been evaluated.¹¹⁾ Among them, autocrine growth factor involvement may best explain estrogen-triggered tumor growth.

MtT/F84, a transplantable pituitary tumor, established and maintained in our laboratory, grows well in 17 β -estradiol-treated rats in a dose-dependent manner.¹²⁾ It was also reported that growth of this tumor is retarded in thyroidectomized rats but can be restored by administration of thyroxine.¹³⁾ In the present study we have examined the effects of retinoic acid on tumor growth, ER level, BrdU incorporation in tumor tissue and serum GH level in tumor-bearing rats.

MATERIALS AND METHODS

Grafting of tumor Four-week-old female F344 rats, purchased from Charles River Co., Tokyo, were ovariectomized and fed with MF basal diet from Oriental Co. Ltd., Tokyo. A week later, tumor cell transplantation was done as described previously.¹²⁾ Tumor cells (10⁵ cells/site) were inoculated sc into 8 sites of breast fat pads of each rat. After grafting, rats were divided into the following groups containing 3-4 rats/group (i.e. grafting sites/group amounted to 24-32). Control rats and rats treated with RA, E, E+tamoxifen, E+RA and E+RA+tamoxifen were implanted with 0.1 mg E₂-pellets (17 β -estradiol, Sigma Chemical Co., E-9000) on the back or/and given RA (all-*trans* retinoic acid, Sigma Chemical Co., R-2625)-enriched diet at doses of 50 or 200 mg/kg of basal diet or/and treated with tamoxifen. Tamoxifen (Sigma Chemical Co., T-9262), suspended in saline containing 1% ethanol, was injected sc into grafted sites at a dose of 20 μ g/10 μ l/site every 3-4 days after cell grafting until termination of the animals. From one week after grafting of tumor cells, grafted sites were checked for tumor growth under mild ether anesthesia every 3-4 days until autopsy. Rats were killed when tumors reached more than 1.0 cm in longest diameter. Part of the tumor tissues was used for BrdU incorporation study and the rest was stored at -80°C for receptor assays.

BrdU incorporation BrdU (Sigma Chemical Co.), dissolved in saline containing 10% dimethylsulfoxide, was injected into the peritoneal cavity 60 min before sacrifice at the dose of 10 mg/100 g body weight. Fresh tumor

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² Abbreviations used: RA, retinoic acid; E, estrogen; tam, tamoxifen; ER, estrogen receptor; RABP, retinoic acid-binding protein; BrdU, 5-bromo-2'-deoxyuridine; RA50 and RA200, rats treated with retinoic acid at dosages 50 and 200 mg/kg basal diet; GH, growth hormone.

tissues were fixed, embedded in paraffin, sectioned and stained for BrdU with mouse monoclonal antibody (Dakopatts, Denmark, Lot 037) using the avidin-biotin complex method. After staining, BrdU-incorporated cells were counted and expressed as a percentage of total number of tumor cells in a selected field.

Preparation of tumor cytosol and nuclear extract Receptor levels were assayed within 2–3 months after tumor collection. Tissues were homogenized in freshly prepared TED buffer (10 mM Tris, 10 mM EDTA, 1 mM dithiothreitol, pH 7.4) at 4°C. Homogenates were centrifuged at 800g for 10 min at 4°C to separate nuclear pellets and supernatants. Supernatants were centrifuged at 90,000g for 60 min at 4°C to get cytosol for assay. Nuclear pellets were washed twice in TED buffer and suspended in KTED buffer (TED buffer containing 0.6 M KCl). Suspended pellets were sonicated for 10–12 s, incubated at 4°C for 60 min and centrifuged at 90,000g for 60 min to obtain nuclear salt extract.

ER assay The procedure was based on that of Bronzert *et al.*¹⁴⁾ and Ginsburg *et al.*¹⁵⁾ Aliquots of cytosol and nuclear extract were incubated with 1–100 nM [³H]E₂ (2,4,6,7-(³H)E₂, 89.8 Ci/mmol, NEN) in the presence or absence of 1,000-fold excess of non-radioactive E₂ (30 μM stock 17β-estradiol) in separate experiments. After 30 min of incubation at 30°C, unbound E₂ was absorbed on dextran-coated charcoal (DCC) and the supernatants were counted. To examine the effects of exogenously added tamoxifen (20 nM) or RA (20 and 100 nM), ER was measured in the presence and absence of both agents. Protein and DNA concentrations of cytosol and nuclear salt extract, before ultracentrifugation, were measured by the methods of Lowry *et al.*, and Labarca and Paigen¹⁶⁾ respectively. Data were plotted according to Scatchard to calculate the maximum number of binding sites per mg protein or DNA.

RABP assay This was performed according to the procedure of Grippo and Gudas.¹⁷⁾ Aliquots of cytosol and nuclear salt extract were incubated with 2–175 nM [³H]RA (11,12-(³H) retinoic acid, 53.4 Ci/mmol, NEN) in the presence or absence of a 600-fold excess of non-radioactive RA (30 μM stock RA) in separate experiments. The amount of alcohol, used to dissolve RA, was kept below 2.6% in the incubation mixture. After incubation at 4°C for 6 h, excess RA was absorbed by the DCC method. Supernatants were counted and data were analyzed to obtain RABP per mg protein or DNA.

GH assay GH levels in sera were determined by a double antibody method.¹⁸⁾ Rat GH (GH-3) as a standard, rat GH (GH-I-5) for iodination and monkey anti-rGH, used in the assay, were supplied by NIADDK. Goat anti-monkey IgG as a second antibody was supplied by Dr. Wakabayashi (Gunma University, Maebashi). Sensitivity of the assay was 0.1 ng/tube. Student's *t*

test was used to determine the statistical significance of differences.

RESULTS

Tumor growth Diet consumption by each rat, both in basal and RA-enriched diet groups, was more or less the same, amounting to 8.0±0.2 g/day/100 g body weight of rat. No tumor was observed until the 12th day after grafting. On day 15 tumor take was 31% in RA50-treated rats and it increased to 100% on day 22 (Table I). In the control group tumor take was 21% on day 22 and reached the maximum of 83% on day 26. Latency, tumor take and tumor weight were significantly increased by RA (*P*<0.01). RA at the dose of 200 mg/kg also affected latency, tumor take and growth similarly to the dose of 50 mg/kg, but no dose-dependency was apparent. In estrogenized rats (0.1 mg/rat), tumor take and latency were not affected but tumor weights were increased in E+RA50-treated and E+RA200-treated rats (*P*<0.01 and 0.05, respectively). Tamoxifen decreased tumor growth in both E- and E+RA-treated groups.

Existence of RABP Tumors contained both cytosolic and nuclear RABP. Maximum numbers of binding sites were 517±62 fmol/mg protein with Kd 4.68±0.91 nM and 250±35 fmol/mg DNA with Kd 2.8±0.95 nM for cytosolic and nuclear RABP, respectively. RABP levels were more or less the same in tumors from different experimental groups (data not shown).

Table I. Effect of E, Tamoxifen and RA on Tumor Latency, Incidence and Weight

Treatment for tumor growth	Latency (day)	Incidence (%)	Weight (g)
Control ^{a)}	22	21	0.62±0.06
"	26	83	—
RA50	15	31	1.02±0.12 ^{b)}
"	22	100	—
E	18	100	1.21±0.53
E+tam	18	100	0.63±0.39
E+RA50	18	100	3.16±1.72
E+RA50+tam	18	100	0.80±0.39
E+RA200	18	100	2.0±0.82 ^{c)}
E+RA200+tam	18	100	0.68±0.26

a) Rats of the control, RA50 and other groups were killed at 30, 30 and 20 days after cell grafting, respectively.

b) The differences of control vs. RA50, E vs. E+tam, E vs. E+RA50, E+RA50 vs. E+RA50+tam and E+RA200 vs. E+RA200+tam are significant (*P*<0.01).

c) The difference of E vs. E+RA200 is significant (*P*<0.05).

Table II. Effect of E, Tamoxifen and RA on ER Level and BrdU Incorporation in MtT/F84

Treatment for tumor growth	Cyt ER ^{a)} (max. binding sites fmol/mg prot.)	Kd (nM)	Nuc ER (max. binding sites fmol/mg DNA)	Kd (nM)	BrdU ^{b)} incorp. (%)
Control	129 ± 23	0.86 ± 0.10	nd ^{d)}	nd	12 ± 2
RA50	187 ± 19 ^{c)}	1.20 ± 0.54	nd	nd	17 ± 3
E	182 ± 16	0.57 ± 0.09	147 ± 29 ^{e)}	0.69 ± 0.26	20 ± 3
E+tam	104 ± 20	0.62 ± 0.15	227 ± 60	0.69 ± 0.22	18 ± 1
E+RA50	401 ± 25	0.80 ± 0.18	258 ± 52	0.30 ± 0.13	35 ± 3
E+RA50+tam	118 ± 31	1.12 ± 0.48	306 ± 18	0.55 ± 0.24	22 ± 4

- a) Each datum for ER, except the control, is the average of 3-4 different tumors.
- b) Each datum for BrdU incorporation is the average of 2-3 different tumors.
- c) The differences of cytosolic ER of RA50 vs. E+RA50, E vs. E+RA50, E vs. E+tam and E+RA50 vs. E+RA50+tam are significant ($P < 0.01$).
- d) Not determined.
- e) The difference of nuclear ER of E vs. E+RA50 is significant ($P < 0.05$).

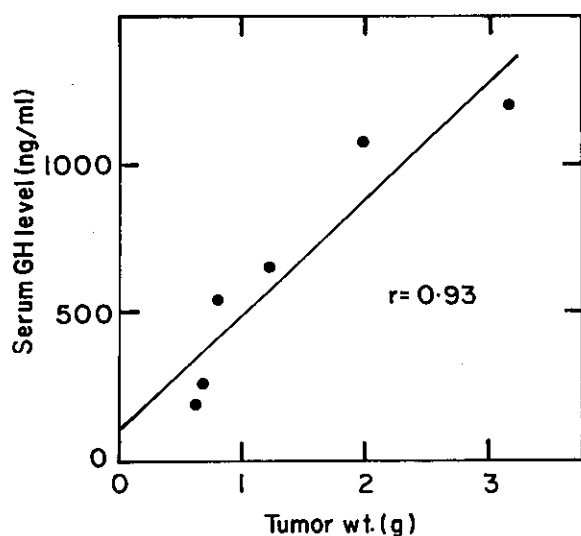


Fig. 1. Correlation between serum GH levels and tumor weights in rats treated with E, E+tam, E+RA50, E+RA50+tam, E+RA200 and E+RA200+tam.

ER levels Table II shows ER levels of tumors from different experimental groups. Cytosolic ER levels were increased in tumors of RA-treated rats when compared with the respective controls. The values were 129 ± 23 , 187 ± 19 , 182 ± 16 and 401 ± 25 fmol/mg protein in the control, RA 50, E, E + RA 50 groups, respectively. Cytosolic ER levels were decreased in tamoxifen-treated rats compared to the respective controls. Nuclear ER level was also elevated in tumors of the E+RA50 group (258 ± 52) compared to that of the E group (147 ± 29).

Table III. Effect of Retinoic Acid and Tamoxifen on Body Weight and Serum GH Level in Rats with MtT/F84

Treatment for tumor growth	Body weight (g)	GH value (ng/ml)
Normal rat serum	—	6.0 ± 2
Control ^{a)}	205 ± 15	2247 ± 393
RA50	216 ± 1	2742 ± 445
E	$156 \pm 5^b)$	$653 \pm 236^c)$
E+tam	139 ± 3	188 ± 57
E+RA50	169 ± 10	1204 ± 509
E+RA50+tam	144 ± 8	538 ± 413
E+RA200	166 ± 3	1079 ± 355
E+RA200+tam	144 ± 8	245 ± 72

- a) Rats of the control, RA50 and other groups were killed at 30, 30 and 20 days after MtT cell grafting, respectively.
- b) The difference of body weight of E vs. E+tam, E+RA50 vs. E+RA50+tam and E+RA200 vs. E+RA200+tam is significant ($P < 0.05$).
- c) The difference of GH of E vs. E+tam, E vs. E+RA50, E vs. E+RA200, E+RA50 vs. E+RA50+tam and E+RA200 vs. E+RA200+tam is significant ($P < 0.01, 0.05, 0.05, 0.05$ and 0.01 , respectively).

An *in vitro* study showed that exogenously added RA at a concentration of 20 nM in the incubation mixture did not affect the ER level, while addition of 100 nM RA affected only cytosolic ER (37% reduction), having no effect on nuclear ER level. Exogenously added tamoxifen also affected the ER level, reducing its value (data not shown). BrdU incorporations in tumor tissues were enhanced by RA (Table II). The scores in tumors grown in

control rats and rats treated with RA50, E and E+RA50 were 12 ± 2 , 17 ± 3 , 20 ± 3 and $35 \pm 3\%$, respectively.

Body weight and serum GH level Table III shows body weights and serum GH levels of MtT/F84-bearing rats. Body weights and serum GH levels were decreased in tamoxifen-treated rats. RA did not have any effect on body weight. Serum GH levels were increased from 653 ± 236 to 1204 ± 509 and from 653 ± 236 to 1079 ± 355 ng/ml in E+RA50-treated and E+RA200-treated rats, respectively. Although body weights in the control and RA50 groups were more or less the same, serum GH level in the RA50 group was higher than that of the control group. Average serum GH level in ovariectomized normal rats was as low as 6 ± 2 ng/ml. Serum GH levels exhibited a good correlation with tumor weights (Fig. 1) under various conditions, which confirmed synthesis and secretion of GH by the tumor.

DISCUSSION

It is clear from the present results that growth of MtT is promoted by RA, as it is by E_2 , although there is no report of estrogenic action of RA. This observation may indicate an intimate relationship between E_2 and RA actions on the growth of the tumor. Recent studies reveal that ER and RAR genes belong to the same steroid hormone receptor gene superfamily¹⁹⁾ and they may have some common functions. It is now believed that RA acts through its binding proteins or receptors^{17, 20, 21)} and they are widely distributed in different tissues including pituitary.²²⁾ We have also confirmed in this report the presence of both cytosolic and nuclear RABP in MtT/F84 using competition binding assay, and no change of RABP level was observed even in RA-treated rats. It seems that change of RABP level is not necessary for tumor growth promotion by RA. BrdU incorporations were also increased both in RA50- and E+RA50-treated tumor tissues compared to the respective controls. Although BrdU incorporations in tumors of the E and E+tam groups are not comparable to tumor growths, the difference between the E+RA50 and E+RA50+tam groups is noteworthy. Thus, tumor growth and BrdU incorporation (i.e. DNA synthesis) in tumor tissue seem to be intimately related.

Our unpublished results suggest that exogenously added RA at physiological concentration (20 nM) *in vitro* does not affect ER determination. RA at a concentration of 100 nM in cytosolic incubation mixture interferes with the ER assay used in this study and decreases the value obtained, but is without any effect on nuclear ER assay. Data on cytosolic ER levels in tumor tissues show that they are increased in RA-treated animals. Nuclear ER level is also increased in tumors of E+RA50-treated rats compared to E alone. It is reasonable to

assume that this increase of ER level in RA-treated rats may be a biological phenomenon rather than a possible interfering effect due to the presence of RA in cytosol or nuclear salt extract. Decrease of cytosolic ER level in tumors of tamoxifen-treated rats may not be a biological phenomenon rather than artifact due to the presence of tamoxifen in the cytosol, since exogenously added tamoxifen (20 nM) in cytosolic incubation mixture reduced its value. It is not clear to us why nuclear ER levels in tumors of tamoxifen-treated rats seem to be higher (though not statistically significant) than in the respective controls. It is now considered that nuclear ER is the functional receptor for E_2 -regulated gene expression^{23, 24)} and our results also show that both cytosolic and nuclear ER levels are increased in tumors of E+RA50-treated rats. This increase of ER levels may be the effect of RA on tumor tissue as a result of which tumor growth is promoted. Elevation of ER level by RA resembles the increase of ER and tumor growth by T_3 ¹³⁾ and the elevation of the progesterone-binding component in uterus cytosols of ovariectomized mouse and rat by estrogen.²⁵⁾ Nevertheless, measurement of both cytosolic and nuclear ER may be important since cytosolic ER level can be interpreted as weak binding of ER to nuclear sites.

Dilution curves of serum GH levels from tamoxifen- and RA-treated rats suggest that tamoxifen and RA which may be present in serum do not have any effect on GH values (unpublished data). Serum GH levels are elevated in RA-treated tumor-bearing rats without any effect on body weight, since the observation period may be too short for the manifestation of the effect of increased serum GH level. Antiestrogenic effects of tamoxifen were also manifested by decrease in body weights and serum GH levels in the E+tam, E+RA50+tam and E+RA200+tam groups. It seems that tamoxifen nullifies the effects of RA on tumor growth and serum GH level as if E_2 and RA have a common mode of action. Serum GH levels in the control and RA50 groups were higher than those in the other groups. This may occur for the following reasons. Firstly, the animals of the control and RA50 groups were killed 30 days after cell grafting and those of the other groups were killed 20 days after cell grafting (see footnote of Table I). Secondly, Inoue *et al.*²⁶⁾ recently isolated four different cell lines from this tumor and each of them has different GH- and PRL-secreting properties and estrogen-dependency. Thus, serum GH levels in rats with tumors grown under different experimental conditions may depend on the population densities of four different cell lines present in a particular tumor. One interesting point is that only GH values from E_2 -treated rats (E, E+tam, E+RA50, etc.) showed a positive correlation with tumor weight (Fig. 1). It is possible to assume that a particular cell line or population density of cell lines is

clonally growing in an E₂-treated state, and tumor growths and serum GH levels differ with slight changes of the conditions (E, E+tam, E+RA50, etc.). Rat pituitary tumors, induced or transplanted, produce and secrete abundant GH in serum.^{18,27)} Morita *et al.*⁶⁾ and Bedo *et al.*⁷⁾ reported that RA promoted synthesis of GH mRNA and secretion of GH in culture media of GH₃ and GH₁ cell lines. The good correlation between tumor weight and serum GH level ($r=0.93$, Fig. 1) in the present study confirms that GH synthesis is also promoted by RA *in vivo*. Although there is a report of alteration of oncogene expression by RA,²⁸⁾ our data allow us to suggest two possible mechanisms to correlate E₂ and/or RA treatment with tumor growth and serum GH level. Firstly, E₂ may promote tumor growth

through its receptor, increasing serum GH level, and RA may act synergistically with E₂ by elevating ER level in the tumor. Secondly, RA may act independently through its binding proteins to promote tumor growth and elevation of serum GH level.

It will be interesting to study whether GH plays any role through its mediator, insulin-like growth factor-I, in the growth of MtT itself. Further studies are necessary to investigate the mechanism by which RA induces elevation of ER and serum GH levels. If RA directly regulates ER and/or GH syntheses in MtT/F84, it may be a good model for the investigation of induction of gene expression by RA.

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