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Specific Triiodothyronine Binding by Tumor Cells and Spleen Cells in a Thyroid Hormone Dependent Mouse Tumor System*

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Abstract—*We have reported thyroid hormone dependency of syngeneic mouse tumor (fibrosarcoma* T241 *in* C57Bl/6J *mice) for growth and spread. To study further the mechanisms of thyroid hormone action we assayed tumor cells and spleen cells for thyroid hormone receptors. Cells were incubated at* 37°C *for* 1 hr *with* [^{125}I]*triiodothyronine with and without unlabeled* T3. *Competitive inhibition of* [^{125}I]T3 *bound to spleen cells was demonstrated by increasing the amount of unlabeled* T3 *and* T4. *Similar competitive inhibition of binding was minimal for intact tumor cells. Saturable binding sites in nuclei were demonstrated in all these cell populations. A Scatchard analysis of nuclei fraction from these cells showed equilibrium dissociation constant* (K_d) *of* 0.56×10^{-9} M *for normal spleen cells and tumor cells and* 1.4×10^{-9} M *for tumor-bearing mice spleen cells. The estimated maximum binding capacity for nuclei of these cells was* 26 fmol/10×10^6 *cells for normal spleen cells,* 46 fmol/10×10^6 *cells for tumor-bearing mice spleen cells and* 45 fmol/10×10^6 *cells for tumor cells. Our results established the presence of* T3 *receptors in nuclei of tumor cells as well as spleen cells and suggests the direct metabolic effect of thyroid hormone as a possible mechanism for thyroid hormone dependency of this tumor.*

INTRODUCTION

THE RELATIONSHIP between thyroid function and cancer has been an issue of controversy for decades. Conflicting results have been reported for both animals and humans [1–8]. We have demonstrated thyroid hormone dependency of syngeneic mouse tumor fibrosarcoma T241 in C57Bl/6J mice [9]. In this system, induced hyperthyroidism (daily injections of exogenous T4 or T3) one month prior to tumor implant enhanced tumor growth and metastases, whereas induced hypothyroidism (^{131}I treatment) inhibited tumor growth and metastases. In an attempt to elucidate the mechanisms of thyroid hormone action we investigated for the presence of specific binding sites of T3 in tumor cells and spleen cells.

We describe our experiments to demonstrate T3 binding by intact tumor cells and spleen cells as well as by subcellular fractions from these cells.

MATERIALS AND METHODS

Mice

Female C57Bl/6J mice (8–10 weeks old) were obtained from Jackson Laboratories, Bar Harbor, ME.

Tumor

Lewis fibrosarcoma (T241), originally induced in C57 mice by dibenzanthracene, was obtained from Sloan Kettering Institute (New York, NY), and the tumor was maintained in our laboratory by serial transfer in these mice.

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T3

Synthetic L-thyroxine and L-triiodothyronine were purchased from Sigma Chemical Company, St. Louis, MO. High specific activity [^{125}I]T3 (specific activity approximately 500 μCi/μg) was obtained from Abbott Laboratories, North Chicago, IL.

Cell preparation

Spleens from normal and tumor-bearing (10–14 days post-tumor implant) C57Bl/6J mice, and tumors from these mice were excised and minced in Hank's balanced salt solution (HBSS). Cell suspensions were then filtered through nylon mesh and the number of cells were counted.

Isolation of nuclear and mitochondrial fractions

The method previously described by Sterling and Milch [10] was used for subcellular fractionations of spleen and tumor cells with slight modifications. Cells (300×10^6) in HBSS were centrifuged (2000 *g* for 10 min) and the pellet was resuspended in 5 ml of 0.25 M sucrose. Cells were then subjected to rapid freezing (liquid nitrogen) and rapid thawing (37°C). This was followed by homogenization with polytron P-10 (3–4 pulses). The homogenate was then centrifuged for 10 min at 2250 *g* in a Sorvall RC-2 refrigerated centrifuge. The pellet consisting primarily of nuclei was washed in Tris–HCl EDTA buffer (10 mM Tris–HCl, 1.5 mM EDTA, 0.5 mM dithiothreitol, pH 7.4, containing 0.5% Triton X-100) and then resuspended in Tris–HCl EDTA buffer and this preparation was used for binding studies. The supernatant (cytosol) was centrifuged at 9000 *g* for 10 min and the pellet, now containing mostly mitochondria with slight microsomal contamination, was resuspended in 0.25 M sucrose and centrifuged at 9000 *g* (repeated twice). The resulting pellet was resuspended in 2 ml of Tris–HCl EDTA buffer containing 0.5% Triton X-100 and incubated for 10 min at 4°C. It was then centrifuged at 140,000 *g* for 1 hr. The resulting supernatant containing mitochondrial membrane protein was used for [^{125}I]T3 binding studies.

Assay for specific T3 binding

Thyroid hormone binding was studied by comparing the [^{125}I]T3 bound to whole cells or by subcellular fractions with and without excess non-radioactive T3. For intact cells, 5 or 10×10^6 cells in 1 ml of RPMI-1640 were incubated in duplicate with 0.5 nM [^{125}I]T3 for 1 hr at 37°C. The tubes were then centrifuged at 2000 rev/min for 10 min. The cell pellet was washed twice with 1 ml 0.9% saline and finally counted in a gamma scintillation counter. Nonspecific binding was assessed by studying this interaction in the presence of excess T3 (10 μM).

For mitochondrial and nuclear fractions, a 0.4 ml aliquot of appropriate dilution (1:4 or 1:8) was incubated with 0.25 nM [^{125}I]T3, with or without excess cold T3. The bound fraction was separated by centrifugation in the case of nuclear fractions, and by dextran-coated charcoal in the case of mitochondrial protein fractions. For the Scatchard plot analysis appropriate cellular fractions were incubated with an increasing amount of [^{125}I]T3 (0.1–2 nM) for 1 hr at 37°C. Again, nonspecific binding was assessed in the presence of cold T3 (2 μM). The bound [^{125}I]T3 was counted and corrected by subtracting nonspecific binding at each concentration. Free [^{125}I]T3 was computed by subtracting corrected bound counts from total counts at each concentration. The bound/free ratio was calculated and plotted against T3 bound for each concentration. All experiments were done in duplicate and each data point represents the mean of two observations.

RESULTS

Normal cells were incubated for different time periods at 37°C in an incubator. Rapid binding of [^{125}I]T3 to cells reaching an equilibrium by 15 min was observed; stable up to 120 min. Relatively high nonspecific binding was observed with tumor cells (64%) as compared with spleen cells (32% for normal spleen, 20% for spleen cells from tumor-bearing animals). Figures 1, 2 and 3 show the ability of cold T3 to compete for the binding sites for normal spleen cells, tumor-bearing cells and tumor cells, respectively. The relative effectiveness of T4 to compete with [^{125}I]T3 binding was studied only with spleen cells from tumor-bearing mice which showed a relative activity of about 0.04 (Fig. 3). Tumor cells demonstrated high capacity binding of [^{125}I]T3 which could only be minimally inhibited by cold T3.

Binding experiments with mitochondrial protein fraction and nuclear fractions demonstrated competitive saturable binding to the nuclear fraction but high capacity nonsaturable binding by the mitochondrial fraction. [^{125}I]T3 binding to mitochondrial protein could not be inhibited by excess cold T3; in fact, the addition of T3 showed enhanced binding (Fig. 4).

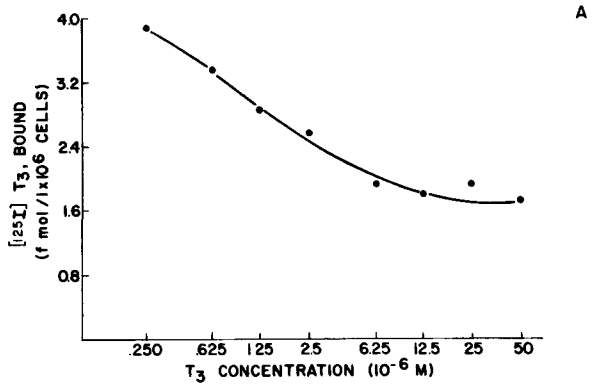

Fig. 1. Competitive inhibition of [^{125}I]T3 *bound to spleen cells from normal mice by nonradioactive* T3. *Cells* (10×10^6) *were incubated at* 37°C *with* 0.5 nM [^{125}I]T3 *for* 1 hr *in the absence and presence of increasing amounts of unlabeled* T3. *Total* [^{125}I]T3 *bound to cells is expressed as* fmol *bound/*1 $\times 10^6$ *cells. The experiment was done in duplicate and each point represents the mean of two observations.*

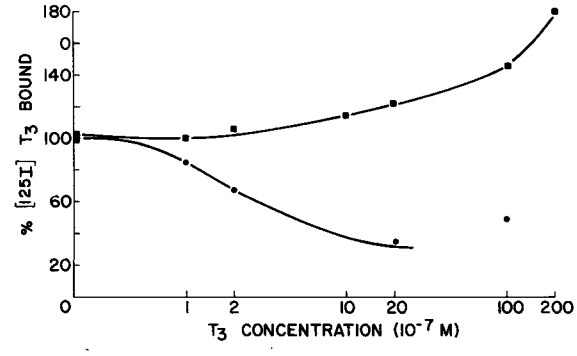

Fig. 4. Competition for [^{125}I]T3 *bound to mitochondrial* (■) *and nuclear fractions* (●) *from spleen cells (tumor bearing mice) by unlabeled* T3. *A* 0.4 ml *aliquot of each fraction was incubated with* 0.5 nM [^{125}I]T3 *for* 1 hr *at* 37°C *in the absence and presence of increasing amounts of unlabeled* T3. *Percentage* [^{125}I] *bound is calculated in relation to the total amount bound in the absence of unlabeled* T3 (100%).

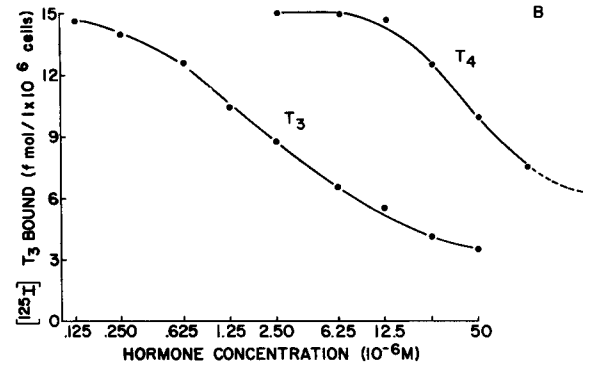

Fig. 2. Competitive inhibition of [^{125}I]T4 *bound to spleen cells from tumor bearing mice by nonradioactive* T3. *The relative effectiveness of* T4 *to compete with* [^{125}I]T3 *binding was also included. Methods as described for Fig.* 1.

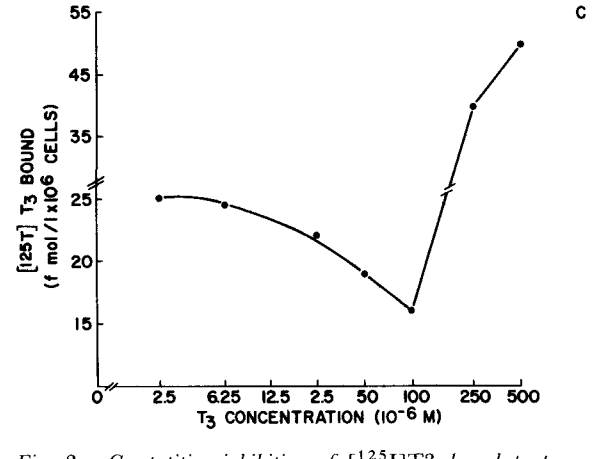

Fig. 3. Competitive inhibition of [^{125}I]T3 *bound to tumor cells by nonradioactive* T3. *Methods as described for Fig.* 1.

Figure 5 shows the Scatchard plot analysis of nuclear binding of [^{125}I]T3 for normal spleen cells, tumor-bearing spleen cells and tumor cells from a single experiment. Two previous experiments with fewer points generated similar numbers. High affinity (K_d, 0.56×10^{-9} M) and low capacity nuclear binding was observed in normal mice spleen cells compared to tumor-bearing mice spleen cells (K_d, 1.4×10^{-9} M). The estimated mean

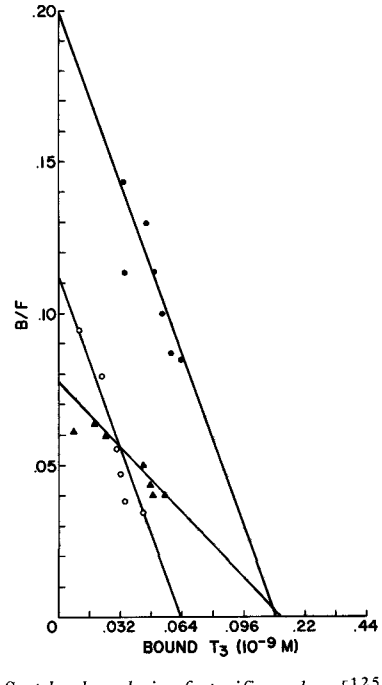

Fig. 5. Scatchard analysis of specific nuclear [^{125}I]T3 *bound by spleen cells from normal mice* (○), *tumor-bearing mice* (▲), *and tumor cells* (●). *Nuclear fraction* (0.4 ml) *was incubated for* 1 hr *at* 37°C *in the presence of increasing* [^{125}I]T3 *concentrations* (0.2–2 nM). *Bound* [^{125}I]T3 *to nuclei was separated by centrifugation followed by two successive washings.*

maximum specific nuclear binding capacity for normal spleen cells was 26 fmol/10×10^6 cells (3200 binding sites/cell), for spleen cells from tumor-bearing mice (10 days post-tumor implant) was 46 fmol/10×10^6 cells (5600 binding sites/cell) and for the tumor cells was 45 fmol/10×10^6 cells (5400 binding sites/cell).

DISCUSSION

Several studies have been undertaken to establish a definite relationship between thyroid function and cancer both in man and experimental animals, but the results are conflicting [1–8]. We have recently described two mouse syngeneic tumor systems, Lewis fibrosarcoma T241 in C57Bl/6J mice and sarcoma 1 in A/Jax mice, which showed clear T4 dependency for tumor growth as well as spread [9]. The results of further experiments with T241 in C57Bl/6J mice reported here established the presence of specific T3 binding sites on the nuclei from tumor cells. This finding favors the possible direct T4 action on the tumor.

The ability of thyroid hormones to stimulate cell proliferation in *in vitro* experiments has been demonstrated previously in two different cancer cell lines [11, 12]. A rat pituitary cell line GH_1 has been shown to respond to thyroid hormone with respect to growth rate [11]. Also, more recently, addition of T3 showed an enhanced proliferation of breast cancer cell line MCF-7 [12]. Both of the above cell lines have been shown to have specific nuclear T3 receptors which possibly explains the T3 action on these cell lines [12, 13]. Several other reports suggest that the presence of nuclear T3 binding sites represents the cellular receptor for thyroid hormones, and binding of T3 to these sites is the first step for the expression of hormonal activity [14–16]. The data, presented here in our system, demonstrate the presence of such specific saturable T3 binding sites on nuclear fraction from tumor cells. This may possibly explain the mechanism of enhanced growth of tumor with T3 and T4 treatment, although *in vitro* experiments to show increased proliferation of tumor cells in relation to increased T3 concentration are required to further evidence this mechanism. In addition, we were also able to demonstrate such T3 binding sites on spleen cells from normal C57Bl/6J mice as well as from tumor-bearing mice.

One of the interesting observations was the significant increase in T3 binding sites in the spleen cells from tumor-bearing mice as compared to those of normal mice spleen cells and a simultaneous decrease of binding affinity. We have previously shown significantly low T4 levels in tumor-bearing mice as compared to the normal mice [9]. The possible mechanism for this increase in T3 receptors in tumor-bearing mice spleen cells could be related to these lower circulating levels of T4 in these mice. The ability of thyroid hormone to modulate its own nuclear receptors have been reported by Samuels and co-workers in GH-1 cells [17]. Also, increased T3 binding capacity by human lymphocytes from hypothyroid patients as compared to euthyroid subjects has been reported [18]. The change in binding affinity due to T3 levels has not been studied so far in any system. It is possible that this decrease in binding affinity is a direct consequence of increase in receptor sites in these cells, although further *in vitro* experiments are required to clarify and confirm these mechanisms. The mechanism by which the growing tumor lowers the serum T4 levels in these mice is not clear and is difficult to explain on the basis of present data. One possibility could be that the high capacity and affinity binding of T3 to tumor cells as observed here is able to alter the metabolism of T3 (faster clearance), which in turn lowers the circulating thyroid hormone concentration in serum. Further experiments to evidence this are obviously required and are on the way in our laboratory.

The mechanism of thyroid hormone action has been studied in rat liver and kidney [19, 20] and the role of nuclear receptors is well established. Specific binding to the mitochondrial membrane protein fraction has been demonstrated in rat liver by Sterling and Milch [10]. In our system, we could not demonstrate specific binding by this fraction, but we observed high capacity unsaturable binding which could not be inhibited by the addition of excess cold T3. Greif and Sloane [21] have reported similar findings in rat liver mitochondria, both in *in vitro* and *in vivo* experiments.

The capability of T4 to compete for T3 binding sites is in agreement with the results of others [12]. As evidenced by competitive binding data, the K_d values in intact spleen cells seems to be about ten-fold lower than observed in isolated nuclei. Whether this is related to the presence of a second low affinity binding site other than nuclei or an artifact of assay conditions is not clear at the present time. Also, the binding observed by intact tumor

cells could only be minimally inhibited by an addition of increasing amounts of unlabeled T3 and in fact, enhancement of binding was observed. A similar increase in binding by excess cold T3 was also observed by Burke and McGuire in breast cancer cell line (MCF-7) [12].

In conclusion, present data established the presence of specific T3 receptors in nuclei from tumor cells (T241) and also in spleen cells. The presence of such receptors strongly suggest the direct metabolic effect of thyroid hormone as a possible mechanism for thyroid hormone dependency of such tumors.

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