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Crucial role of thyroid hormone in x-ray-induced neoplastic transformation in cell culture

(triiodothyronine/mouse embryo cells/transformation *in vitro*/irradiation/protein synthesis)

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ABSTRACT Incubation of mouse embryo fibroblasts (C3H/10T½) in media depleted of thyroid hormone for 1 week rendered the cells completely resistant to the transforming action of an x-ray dose, 4 grays, that yields transformation frequencies (no. foci per surviving cells) of $\approx 10^{-3}$ in media supplemented with triiodothyronine (T3) (1 nM). Studies on the timing of the additions or removal of the hormone indicate that T3 was maximally effective when added 12 hr before irradiation and that progression from the time of irradiation to the appearance of foci (6 weeks) was independent of the presence or absence of the hormone. The dependence of x-ray-induced transformation on the concentration of T3 in the medium was virtually the same as that for augmentation of Na^+,K^+-ATPase activity. The latter effect was used as a measure of T3 induction of protein synthesis. A further indication of the involvement of protein synthesis in the process is the abolition of T3- and x-ray-dependent transformation by cycloheximide at a concentration (100 ng/ml) that inhibits 50% of protein synthesis. We propose that thyroid hormone induces the synthesis of a host protein that is an obligatory participant in x-ray-mediated transformation.

Guernsey *et al.* (1) found that removal of thyroid hormones triiodothyronine (T3) and thyroxine (T4) from serum-supplemented media eliminated x-ray-induced neoplastic transformation of both hamster embryo cells (primary or secondary cultures) and C3H/10T½ cells without modifying their survival or growth rates. Moreover, addition of 0.1 μM T3 to thyroid hormone-depleted medium reestablished the expected frequency of transformation.

In the present report we provide further details on the crucial role of thyroid hormone in x-ray-induced neoplastic transformation of C3H/10T½ cells including delineation of the time when transformation is sensitive to T3, dependence of transformation on T3 concentration, and the involvement of protein synthesis. The results indicate that thyroid hormone plays a key role in the initiation of x-ray-induced neoplastic transformation and that induction of protein synthesis may mediate this response.

MATERIALS AND METHODS

Materials. AG1-X10 resin, chloride form, was purchased from Bio-Rad, 3,3′,5′-triiodo-L-thyronine was from Calbiochem, and other chemicals were from Sigma. Heat-inactivated fetal calf serum, Eagle's medium, and antibiotics were obtained from GIBCO; [^{35}S]methionine (700 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was from Amersham.

Cell Cultures and Conditions. C3H/10T½ (clone 8) mouse embryo fibroblast cells, originally obtained from C. Heidelberger, were treated as described (2). Stock cultures were maintained at 37°C, aerated with 5% CO_2 in air, in Eagle's basal medium containing 10% heat-inactivated fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml). T4 and T3 were removed from the fetal calf serum as described by Samuels *et al.* (3) by adsorption to AG1-X10 resin. Stock 1 mM T3 in 50% *n*-propanol was diluted with medium supplemented with 10% resin-treated fetal calf serum to give the final concentration desired. Media depleted of thyroid hormones were prepared with 10% resin-treated fetal calf serum and an amount of diluent equal to that added to thyroid-supplemented media. Cells were incubated in T3-supplemented (+T3) or T3-depleted (−T3) media for 1 week prior to exposure to x-irradiation. The cells were reseeded and 24 hr later were x-irradiated with either 3 or 4 grays (1 gray = 100 rads) at room temperature at a dose rate of 0.42 gray/min as described (1). Six weeks after irridation (with weekly media changes), the cells were fixed and stained with Giemsa and scored for transformation as described by Reznikoff *et al.* (4). Both type II and III foci were scored as transformed. Plating efficiency and surviving fraction of cells following irradiation were established as described (5).

Na^+,K^+-ATPase Assay. Growth-arrested cell cultures at confluency were rinsed twice with Dulbeccos' phosphate-buffered saline; then 10 ml media containing resin-treated fetal calf serum and various concentrations of T3 were added to the cultures. The media were removed 4 days later, and the cells were rinsed twice with phosphate-buffered saline and harvested with a rubber policeman. A cell pellet was obtained by centrifuging at $800 \times g$ for 5 min and was stored at −70°C for up to 2 weeks.

The frozen cell pellet was thawed quickly, resuspended in 0.25 M sucrose/30 mM Tris/0.1% deoxycholic acid, pH 7.2, and sonicated with a microtip in an ice-bath with three 7-sec pulses at setting 6 (Sonifier Cell Disruptor, Heat Systems/Ultrasonics, Plainview, NY), interrupted by 10-sec cooling periods. The sonicates (100-μl aliquots) were assayed for Na^+,K^+-ATPase activity in 900 μl of 120 mM NaCl/20 mM KCl/3 mM $MgCl_2$/1 mM EDTA/30 mM Tris/5 mM NaN_3/3 mM Tris ATP, pH 7.4, at 20°C. Tandem assay tubes contained 1 mM ouabain in the assay media. The tubes (in triplicate) were incubated for 15 min at 37°C and the reaction was terminated by the addition of 30% (vol/vol) ice-cold trichloroacetic acid and immersion of the tubes in an ice-water bath. After 40 min on ice, the mixture was centrifuged at $10,000 \times g$ for 4 min and the P_i content of the supernatant was determined by the method of Fiske and Subbarow (6). Protein content of each precipitated pellet was determined by the method of Lowry *et al.* (7). Na^+,K^+-ATPase activity was calculated as the difference in the

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Abbreviations: T3, triiodothyronine; T4, thyroxine.

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activities obtained without and with ouabain in the assay mixture.

Cycloheximide: Cell Survival and Protein Synthesis. For assay of survival, cells were seeded at a density of ≈7.0 × 10^5/10-cm dish; 24 hr later (time 0), the dishes contained ≈2.0 × 10^5 surviving cells. At time 0, cycloheximide was added to groups of culture dishes to give final concentrations of 0, 1, 10, 100, 1000, 10,000, or 100,000 ng/ml. Thirty-six hours later the total number of attached cells per plate was counted.

To study protein synthesis, 10-cm dishes were seeded with 1.5 × 10^6 cells per dish; 24 hr later (time 0), the dishes contained 4.0 × 10^5 surviving cells per dish. Cycloheximide was then added to groups of dishes to final concentrations of 1, 10, 50, 100, and 1000 ng/ml. One hour after cycloheximide addition, 30 μCi of [^{35}S]methionine was added to each dish and the dishes were incubated at 37°C in 5% CO_2 in air for 1 hr. The medium was then removed and the dishes were rinsed extensively with phosphate-buffered saline. Each plate then received 2 ml of buffered saline with 0.5% Nonidet P-40 (British Drug House, Pooler, England) in 1 mM EDTA. After the cells were detached from the dish, the cell suspension was sonicated three times at setting 6 for 10 sec with 10-sec cooling periods. To 1 ml of sonicate was added an excess of unlabeled methionine. The solution was vortexed well and 0.2 ml of 30% trichloroacetic acid was added. The solution was kept at 4°C for 30 min. One milliliter of the precipitate mixture was put on 2.4-cm GFC filters (Whatman) with suction and the filters were washed extensively with 5% trichloroacetic acid under continuous suction. The filters were assayed for ^{35}S.

RESULTS

X-ray induced transformation and cell survival of C3H/10T½ cells were assessed in media supplemented with 10% resin-treated fetal calf serum with or without added T3. As reported (1), the removal of thyroid hormone from the medium (by resin-treatment of the serum) completely suppressed x-ray induced transformation (Table 1). When T3 was added to the media (final concentration, 0.1 μM), the transformation frequency was ≈2 × 10^{-4}. This frequency of transformation is similar to that obtained with either resin-treated fetal calf serum supplemented medium with added T3 or with medium supplemented with unmodified fetal calf serum in our earlier study (1). Cells incubated in T3-supplemented media for 1 week prior to irradiation and then transferred to T3-deficient medium at the time of irradiation (time 0) exhibited only a slight decrease in the frequency of transformation. Moreover, T3 pretreated cells transferred to deficient media 24 hr after irradiation and maintained in the thyroid-depleted state for 6 weeks transformed at the same frequency as the group exposed continuously to T3 throughout the entire 7 weeks of the experiment. These results indicate that the effect of T3 is exerted at the time of irradiation or soon thereafter, and that thyroid status plays little or no role beyond initiation and fixation of radiation-induced transformation. This was confirmed by the finding that preincubation in T3-depleted media for 1 week and supplementation with T3 for 12 hr before irradiation restored transformation frequency to the same rate as in the cells pretreated with T3 for 1 week. When T3 was added at the time of irradiation the transformation frequency was diminished; no transformation was observed when T3 was added 12 hr or more after irradiation.

The dependence of x-ray-induced transformation on the concentration of T3 in the medium is shown in Fig. 1. Maximal transformation frequency was exhibited with cells exposed to 0.1 nM T3. The concentration–response relationship exhibited a $K_{1/2}$ of 10 pM T3. Fig. 1 also demonstrates the close similarity in the concentration-dependence of the induction of Na^+,K^+-ATPase and of transformation. Induction of Na^+,K^+-ATPase was maximal at 1 nM T3, with a $K_{1/2}$ of 60 pM. In view of the evidence indicating that T3 regulates Na^+,K^+-ATPase activity by inducing *de novo* synthesis of the subunits (8, 9), these results as well as the time-course data raise the possibility that T3 induces the synthesis of host protein(s) that are involved in the process of transformation. Further information on this possibility was obtained with reverse T3, an inactive isomer of the native hormone (9, 10), and with cycloheximide, a potent inhibitor of protein synthesis (11, 12).

As in the earlier trials (1), cells depleted of T3 were completely insensitive to x-ray induction of neoplastic transformation *in vitro* (Tables 1 and 2; Fig. 1). Cells exposed to T3 (1 nM) 12 hr before irradiation and grown in T3-depleted media 24 hr after irradiation transformed at the maximum frequency (≈10^{-3}). In contrast, supplementation with reverse T3 (1 nM) for the same interval yielded no transformations. Moreover, cycloheximide at 50 ng/ml added and removed from the media concurrently with T3 (i.e., 12 hr before and removed 24 hr after irradiation) suppressed transformation frequency by 40%. This concentration and time of exposure to cycloheximide result in 71% cell survival and inhibit cellular protein synthesis by 20% (Table 3). Exposure to cycloheximide at 100 ng/ml concurrently with T3 (i.e., same 36-hr period) inhibited transformation en-

Table 1. Time-dependence of thyroid hormone modulation of x-ray-induced neoplastic transformation *in vitro*

	Medium					
Pretreatment	At 12 hr before x-ray	At x-ray	At 12 hr after x-ray	At 24 hr after x-ray and thereafter for 6 weeks	Transformed foci/ surviving cells	Transformation frequency (foci/surviving cells)
+T3	+T3	+T3*	+T3	+T3	0/27,168†	—
−T3	−T3	−T3*	−T3	−T3	0/27,160†	—
+T3	+T3	+T3	+T3	+T3	4/21,746†	1.8 × 10^{-4}
−T3	−T3	−T3	−T3	−T3	0/35,760†	—
+T3	+T3	−T3	−T3	−T3	1/9,450	1.1 × 10^{-4}
+T3	+T3	+T3	+T3	−T3	6/12,600	4.7 × 10^{-4}
−T3	+T3	+T3	+T3	+T3	5/11,210	4.5 × 10^{-4}
−T3	−T3	+T3	+T3	+T3	2/23,570†	8.5 × 10^{-5}
−T3	−T3	−T3	+T3	+T3	0/12,750	—
−T3	−T3	−T3	−T3	+T3	0/16,480†	—

T3 was at 0.1 μM when present. X-irradiation was at 3 grays.
* No x-irradiation.
† Composite of two separate experiments.

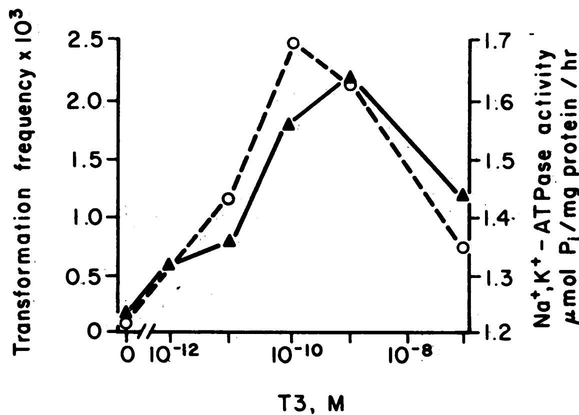

FIG. 1. Effect of varying concentrations of T3 on transformation frequency (○) and Na^+,K^+-ATPase activity (▲). For transformation experiments cells were pretreated in various doses of T3 for 1 week prior to irradiation (4 grays) and maintained in the same conditions for the remainder of the experiment.

tirely (Table 2). At this higher dose of cycloheximide, approximately 45% of the cells survived the 36-hr exposure to the inhibitor, and protein synthesis was inhibited by 50%.

DISCUSSION

Studies by Borek and Sachs (13–15) indicated that mammalian cell transformation by radiation is composed of early steps when initiation and fixation of the transformed state takes place as well as later ones whereby the transformed phenotype is expressed. The present report indicates that thyroid hormone plays a critical role in early events of x-ray-induced neoplastic transformation. This inference is based upon the complete inhibition of x-ray-induced transformation when the cells are cultured in medium free of thyroid hormone and the complete restoration of sensitivity to transformation by addition only of T3 to the medium, 12 hr prior to irradiation.

To deplete the cells of thyroid hormone, the fetal calf serum used in the culture medium was treated with an anion exchange resin. An important issue is the specificity of this procedure and the possibility of extraction of other regulatory agents. The available evidence supports the inference of selective depletion of T3 and T4 in that low molecular weight organic anions are depleted only minimally, cortisol concentration (as a model steroid) is decreased to a relatively small degree, and the total protein content and electrophoretic patterns of serum proteins

Table 2. Effect of T3, rT3, and T3 + cycloheximide on x-ray-induced neoplastic transformation *in vitro*

Pretreatment	Medium 12 hr before x-ray	Medium 24 hr after x-ray	Transformed foci/surviving cells	Transformation frequency
−T3	+T3	−T3*	0/8,820	—
−T3	+T3	−T3	14/15,040	9.3×10^{-4}
−T3	−T3	−T3	0/15,130	—
−T3	+rT3	−T3	0/16,005	—
−T3	+T3†	−T3	7/12,354	5.7×10^{-4}
−T3	+T3‡	−T3	0/13,200	—

T3 was at 1 nM when present; rT3, reverse T3 at 1 nM. Irradiation was 4 grays.
* No x-irradiation.
† Plus cycloheximide at 50 ng/ml.
‡ Plus cycloheximide at 100 ng/ml.

Table 3. Effect of varying doses of cycloheximide on cell survival and protein synthesis

Cycloheximide, ng/ml	Cell survival, %	Protein synthesis, %
0	100	100
1	100	ND
10	100	100
50	71	80
100	47	53
1,000	35	29
10,000	9	ND
100,000	6	ND

Cell survival was based on a 36-hr exposure to the cycloheximide. Protein synthesis data were determined after a 2-hr exposure to cycloheximide with [^{35}S]methionine included in the second hour. ND, not done.

are unchanged (3). Resin treatment reduces T3 content of calf serum from 150 to 2.0 ng/dl and T4, from 6.8 to 0.08 μg/dl. Samuels *et al.* (3) further showed that in GH_1 cells in culture, resin-treated calf serum gave biological responses identical to those obtained with thyroidectomized calf serum. In studies to be reported elsewhere, we found that C3H/10T½ cells also exhibit the same responses to T3 (i.e., the induction of Na^+,K^+-ATPase and cytochrome *c* oxidase) in culture media supplemented with either resin-treated fetal calf serum or thyroidectomized calf serum.

Addition of T3 to the medium just 12 hr before irradiation and removal 12 hr after irradiation elicits full expression of transformation. Moreover, the addition of T3 12 or 24 hr after irradiation yields no transformants even though the cells were maintained in T3 supplemented media for the entire 6 weeks required for expression of the transformed state. It is important to note that, when T3 is added at the time of irradiation, there is a dramatic reduction (80%) in transformation frequency. These results indicate that T3 is required only during initiation of x-ray-induced transformation but not at the later phase of expression. Furthermore, it is evident that the critical period for the presence of thyroid hormone in the culture medium is the 12-hr period just prior to irradiation.

An important characteristic of thyroid hormone action is the lag time of 8–24 hr between the administration of the hormone and the augmentation of protein synthesis (10, 16). The time dependence of the action of T3 on transformation is consistent with a possible induction of a host protein that acts in concert with the events set in motion by irradiation.

The dependence of x-ray-induced transformation on T3 concentration falls within the range of hormone levels in the circulation of intact animals under physiological conditions (10, 17). Maximal transformation frequency was observed at a T3 concentration of 0.1 nM with a $K_{1/2}$ of 10 pM. The concentration-dependence of T3 induction of the cell membrane-associated Na^+,K^+-ATPase is similar to the T3-augmentation of transformation (Fig. 1). Considerable evidence has accumulated indicating that thyroid hormone regulates this enzyme *in vivo*, via nuclear receptors and induction of the coordinate synthesis of both subunits (8, 9). It is important to note that thyroidal augmentation of Na^+,K^+-ATPase in C3H/10T½ cells was elicited in the same media (resin-treated fetal calf serum with and without) as was T3 modulation of neoplastic transformation. Moreover, both responses to T3 (Na^+,K^+-ATPase and x-ray-induced transformation) appear to have similar latent periods (i.e., 12–24 hr). These analogies raise the possibility that thyroid hormone induces a host protein that mediates irradiation-induced trans-

formation. This inference is supported by the finding that T3 modulation of x-ray-induced transformation was suppressed by cycloheximide at 50 ng/ml and eliminated at 100 ng/ml. The latter concentration inhibits protein synthesis by 50% in C3H/10T½ cells. Additionally, L-3,3'-5'-triiodothyronine (reverse T3), an isomer without effect on protein synthesis (9, 10), had no effect on neoplastic transformation (Table 2). Thus, the findings to date merit further studies on the postulated thyroid-induced pathways mediating experimental carcinogenesis and on the possibility that these hormones play a significant role in carcinogenesis in man.

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