Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 5749–5752, September 1983
Medical Sciences

Critical role played by thyroid hormone in induction of neoplastic transformation by chemical carcinogens in tissue culture

(triiodothyronine/*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine/benzo[*a*]pyrene/cell cultures/protein synthesis)

CARMIA BOREK*†, DUANE L. GUERNSEY‡§, AUGUSTINUS ONG†, AND I. S. EDELMAN‡

Departments of †Radiology, *Pathology, and ‡Biochemistry, College of Physicians and Surgeons of Columbia University, New York, New York 10032

Contributed by I. S. Edelman, June 20, 1983

ABSTRACT **Incubation of primary cultures of hamster embryo cells (HEC) or mouse fibroblasts (C3H/10T$^1/_2$ cells) in media depleted of thyroid hormones does not alter cell growth or survival but renders the cells resistant to neoplastic transformation by benzo[*a*]pyrene (B[*a*]P) or *N*-methyl-N'-nitro-*N*-nitrosoguanidine (MNNG), carcinogens which yield transformation rates of 10^{-4}–10^{-2} in media supplemented with triiodothyronine (T3). In C3H/10T$^1/_2$ cells, the times of addition or removal of the hormone indicate that T3 exerts maximum effect when added 12 hr prior to treatment with B[*a*]P and that the progression of transformation from the time of initiation by the carcinogen to full expression and the appearance of transformed foci was independent of the presence or absence of the hormone in the medium. Dependence of transformation on T3 concentration in the medium was observed over the physiological range of 1 pM to 100 nM in C3H/10T$^1/_2$ cells treated with B[*a*]P. These results were similar to our previous findings on the T3 dose-related induction of radiogenic transformation and of Na^+,K^+-ATPase activity. The latter effect was used as a measure of T3 induction of protein synthesis. A further indication of the potential involvement of protein synthesis in T3 action is the suppression of T3- and B[*a*]P-dependent transformation by cycloheximide at concentrations that inhibit protein synthesis by ≈50% in the C3H/10T$^1/_2$ cells. We suggest that thyroid hormone induces the synthesis of a host protein that plays a key role in neoplastic transformation by direct-acting chemical carcinogens and by those requiring metabolic activation. In our previous studies, similar T3-dependent mechanisms were implicated in radiogenic transformations.**

Our earlier studies, with primary cultures of hamster embryo cells (HEC) and mouse embryo fibroblasts (C3H/10T$^1/_2$), an established line, indicated that thyroid hormones are essential factors in the induction of neoplastic transformation by x-irradiation (1, 2). Removal of triiodothyronine (T3) and thyroxine (T4) from serum-supplemented media eliminated radiogenic transformation in both cellular systems without modifying the rates of cell survival or cell growth (1, 2). The addition of T3 (1 pM to 100 nM) to the thyroid hormone-depleted medium reestablished transformation at frequencies that were T3 dose dependent. The T3 concentration dependence of the transformation response to x-rays was the same as the concentration dependence of the Na^+,K^+-ATPase response (2).

In these earlier studies (2), the maximal transformation frequency was observed when T3 was added to the medium 12 hr prior to x-ray treatment. In contrast, when T3 was added to the hormone-depleted cells 24–48 hr after exposure to x-rays, no transformation was observed. These findings strongly suggested that thyroid hormones serve as crucial factors in the initiation of cellular neoplastic transformation by x-irradiation.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

The present experiments were undertaken to assess the role of thyroid hormone in neoplastic transformation induced by specific chemicals in both the diploid HEC and the heteroploid C3H/10T$^1/_2$ strains as the *in vitro* model systems (3–5).

We report here that, in these two culture systems, the presence of thyroid hormone, specifically T3, is crucial for the induction of transformation by benzo[*a*]pyrene (B[*a*]P), which requires metabolic activation, and by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), an alkylating agent that is a direct-acting carcinogen (6, 7).

Our results indicate that T3 plays a key role in the initiation of neoplastic transformation by B[*a*]P and MNNG and that intact protein synthesis is required for these responses.

MATERIAL AND METHODS

AGI-X10 resin (chloride form) was purchased from Bio-Rad; 3,3',5'-triiodo-L-thyronine, from Calbiochem; and B[*a*]P, from Sigma. Heat-inactivated fetal calf serum, Eagle's basal medium, and antibiotics were obtained from GIBCO, and MNNG was from Aldrich. All of the conventional reagents were of analytical grade or of the highest purity available.

Cell Cultures and Conditions. C3H/10T$^1/_2$ (clone 8) cells were originally obtained from C. Heidelberger. 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) (4, 5). HEC were obtained by mincing midterm whole embryos from Golden Syrian hamsters (Lakeview, Wilmington, MA). Primary cultures were established by progressive dissociation of minced fresh tissue in Dulbecco-modified Eagle's medium fortified with 10% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml) (3, 5).

T3 and T4 were removed from fetal calf serum by adsorption to AGI-X10 resin as described (1, 2, 8). Stock T3 (1 mM in 50% 1-propanol) was diluted with medium supplemented with 10% resin-treated fetal calf serum to give the final concentration desired. Medium depleted of thyroid hormones was prepared with 10% resin-treated fetal calf serum and an amount of diluent equal to that added to the T3-supplemented medium. Cells were incubated in T3-supplemented or T3-depleted medium for 1 week prior to exposure to one of the carcinogenic agents. The cells were reseeded, and 24 hr later the C3H/10T$^1/_2$ or HEC cells were treated with B[*a*]P (1.2 μg/ml). The cells were exposed to the chemical for 48 hr, at which time the medium was

Abbreviations: T3, triiodothyronine; T4, thyroxine; Na^+,K^+-ATPase, Na^+,K^+-activated adenosine triphosphatase (EC 3.6.1.3); B[*a*]P, benzo[*a*]pyrene; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; HEC, hamster embryo cells; rT3, reverse T3.

§ Present address: Dept. of Physiology and Biophysics, Univ. of Iowa College of Medicine, Iowa City, IA 52242.

removed, the cells were washed with Hanks' balanced salt solution, and fresh medium was added. The T3-free or -supplemented conditions were maintained for the duration of the experiment. In another experiment the C3H/10T$^1/_2$ and HEC cells were treated with MNNG at 0.5 μg/ml for 4 hr.

To determine the dependence of B[*a*]P-induced transformation on the concentration of T3 in the medium, the C3H/10T$^1/_2$ cells were incubated at selected concentrations of T3 for 1 week prior to the reseeding of the cells and to their exposure to the chemical for 48 hr. The selected concentrations of T3 also were maintained for the duration of the experiment.

To define the time-dependence of T3 modulation of chemically induced transformation, the C3H/10T$^1/_2$ cells were incubated with T3-free or -supplemented medium for 1 week prior to reseeding. Twelve hours before exposure to B[*a*]P, all media were changed. In two sets of experiments, cells previously incubated in T3-free medium were incubated in T3-supplemented medium, and in the remainder T3-free conditions were maintained. At time zero the chemical carcinogen was added to the media, and 48 hr later all media were replaced with fresh, untreated media. In one set of experiments in which T3 had been added to the medium 12 hr before exposure to the carcinogens, T3-free medium was substituted and used for the remainder of the test period.

Transformation Assay. Six weeks after exposure to the carcinogen (during which time the media were changed weekly) the C3H/10T$^1/_2$ cells were fixed and stained as described by Reznikoff *et al.* (4). Both type II and III foci were scored as transformed. Plating efficiency and surviving fraction of cells after treatment were evaluated as described (4). For the HEC cells, 2 weeks after the chemical treatment the cells were fixed and stained with Giemsa (3). A differential count was made of normal and transformed colonies, the latter being identified by their morphological appearance among the surviving colonies. Both normal and transformed colonies were scored so that both transformation frequency and cell survival could be assessed within the same experiment. Transformed colonies were identified by their irregular growth pattern and their tendency to form multilayers as compared to controls (3, 5).

RESULTS

In the presence of unmodified fetal calf serum (10%), the yield of transformants in response to a B[*a*]P in C3H/10T$^1/_2$ cells was $\approx 10^{-3}$ (Table 1). The removal of thyroid hormones from the media by resin treatment of the serum resulted in a complete suppression of neoplastic transformation by the B[*a*]P. Addition of T3 (2 nM) to the resin-treated medium (the cells were grown and maintained in the T3-fortified medium) restored the rate of transformation induced by B[*a*]P to that obtained in the presence of unmodified fetal calf serum.

With unmodified fetal calf serum, the direct carcinogen MNNG elicited a transformation frequency of 3×10^{-3} in C3H/10T$^1/_2$ and 1.4×10^{-2} in HEC (Table 2). Depletion of T3 and T4 from the media (i.e., with resin-treated fetal calf serum) eliminated all transformations in both types of cells, and readdition of T3 (2 nM) to the media restored the transformation frequencies to the control values.

Table 1. The effect of thyroid hormone on B[*a*]P-induced *in vitro* neoplastic transformation of C3H/10T$^1/_2$ cells*

Serum treatment†	Transformed foci per surviving cell	Transformation frequency
Eu	0/16,958	0
Eu + B[*a*]P	10/11,350	8.8×10^{-4}
−T3 − B[*a*]P	0/15,000	0
−T3 + B[*a*]P	0/12,680	0
+T3 + B[*a*]P	14/17,872	7.8×10^{-4}

* B[*a*]P was added at 1.2 μg/ml for a treatment period of 48 hr.
† Eu, untreated fetal calf serum; −T3, resin-treated fetal calf serum depleted of thyroid hormones; +T3, resin-treated serum fortified with 2 nM T3.

In our earlier studies on the dependence of x-irradiation-induced transformation on T3 in the medium, we found that T3 was required only for a 36-hr interval (i.e., from 12 hr before to 24 hr after irradiation), implying no effect of the hormone on progression of the process (2). Accordingly, similar studies were carried out on the time relationships in the conjoint action of T3 and B[*a*]P. As indicated by the first two rows in Table 3, in the continuous presence of resin-treated serum, the presence or absence of B[*a*]P elicited no transformants. Preincubation of the cells for 1 week in resin-treated serum, followed by addition of T3 (1 nM) for 12 hr prior to B[*a*]P treatment restored transformation rates in C3H/10T$^1/_2$ cells to those observed in cells pretreated with T3 for 1 week (cf. row 4 in Table 1 and row 3 in Table 3). Addition of T3 to the medium simultaneously with B[*a*]P reduced the yield of transformants to 40% of the optimum (row 4) and addition 48 hr after B[*a*]P addition (i.e., at the time of removal of this agent) was totally ineffective in eliciting transformation (row 5). Moreover, when T3 was added 12 hr prior to addition of B[*a*]P and both agents were removed 48 hr thereafter, transformation rates were restored to optimal values (row 6). In fact, exposure to T3 for only the 12 hr before B[*a*]P was added (i.e., replacement with resin-treated serum at the time of addition of B[*a*]P) was sufficient to elicit about 1/3 of the control transformation frequency (row 7). These results agree closely with the earlier findings with x-irradiation (2).

The dependence of B[*a*]P-induced transformation on the concentration of T3 in the medium is compared in Fig. 1 to similar earlier observations on radiogenic transformation and induction of Na^+,K^+-ATPase (9). The similarities in these responses are striking. As with radiogenic transformation and augmentation of Na^+,K^+-ATPase activity, the transformation

Table 2. The effect of thyroid hormone on MNNG-induced neoplastic transformation in HEC and C3H/10T$^1/_2$ cells*

Serum treatment	C3H/10T$^1/_2$		HEC	
	Transf. foci/ surviving cells	Transf. frequency	Transf. colonies/ surviving cells	Transf. frequency
Eu	0/17,501	0	0/4,125	0
Eu + MNNG	37/12,300	3.0×10^{-3}	30/2,120	1.4×10^{-2}
−T3 + MNNG	0/13,100	0	0/5,780	0
+T3 + MNNG	21/11,305	1.9×10^{-3}	34/2,614	1.3×10^{-2}

* MNNG was added at 0.5 μg/ml for a treatment period of 4 hr.
† Eu, untreated fetal calf serum; −T3, resin-treated fetal calf serum depleted of thyroid hormones; +T3, resin-treated fetal calf serum fortified with 2 nM T3.

Table 3. Time dependence of thyroid hormone modulation of B[a]P-induced neoplastic transformation in C3H/10T$^1/_2$ cells*

T3-free medium				
12 hr before B[a]P	At B[a]P exposure	48 hr after B[a]P	Transformed foci per surviving cells	Transformation frequency
−T3	−T3†	−T3	0/9,620	0
−T3	−T3	−T3	0/13,528	0
+T3	+T3	+T3	17/10,320	1.6×10^{-3}
−T3	+T3	+T3	8/12,152	6.6×10^{-4}
−T3	−T3	+T3	0/13,000	0
+T3	+T3	−T3	13/11,220	1.2×10^{-3}
+T3	−T3	−T3	5/12,032	4.2×10^{-4}

*T3 was at 1 nM when present; B[a]P was added at 1.2 μg/ml and removed after 48 hr of treatment.
†No B[a]P was added.

response was biphasic with a maximum at ≈1 nM T3. These results and the time-dependence data summarized in Table 3 suggest that T3 acts as a permissive factor by induction of the synthesis of host protein(s). To explore this possibility, further studies were done with reverse T3 (rT3), an isomer with no known inducing activity (10, 11), and with cycloheximide, a potent inhibitor of translation (12).

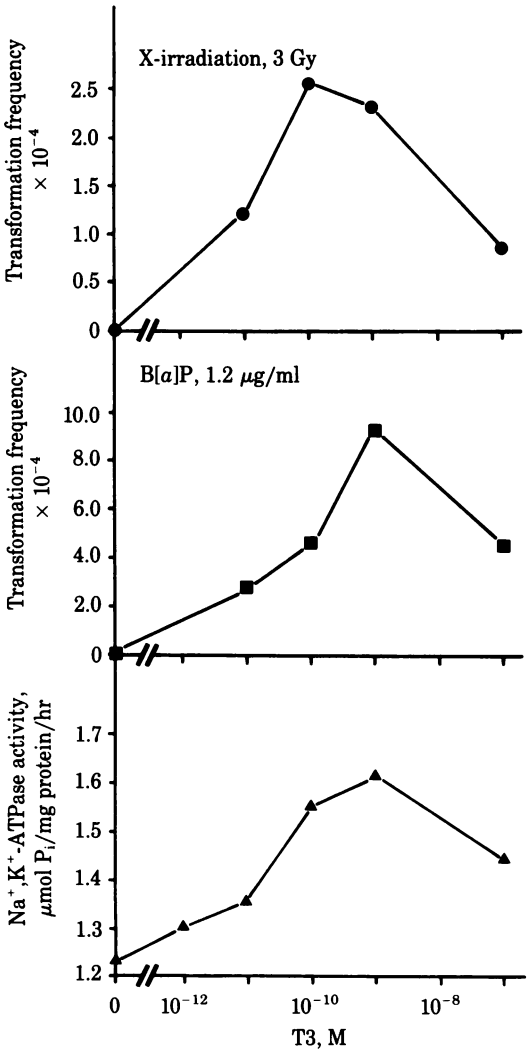

FIG. 1. The effect of varying concentrations of T3 on x-ray-induced (●) and B[a]P-induced (■) transformation and on Na$^+$,K$^+$-ATPase activity (▲) in stationary-phase C3H/10T$^1/_2$ cells. For transformation experiments, cells were pretreated with various doses of T3 for 1 week prior to treatment with B[a]P (1.2 μg/ml) or x-rays (4 Gy) and maintained under the same conditions for the remainder of the experiment. Data on x-rays and Na$^+$,K$^+$-ATPase are from Guernsey *et al.* (2).

Cells grown in resin-treated medium and then exposed to medium supplemented with rT3 12 hr prior to B[a]P treatment yielded no transformants in contrast to cells exposed to medium fortified with T3 either throughout the experiment or for 12 hr before to 48 hr after exposure to B[a]P (cf. rows 3, 4, and 5 in Table 4). In previous experiments on C3H/10T$^1/_2$ cells, exposure to cycloheximide for 36 hr at 50 ng/ml inhibited overall protein synthesis by 20%, accompanied by 71% cell survival (2). At a cycloheximide concentration of 100 ng/ml, protein synthesis was inhibited by 50%, and 45% of the cells survived. In the present experiments with cycloheximide at 50 ng/ml, the yield of transformants in response to T3 and B[a]P was reduced to 15%, and at 100 ng/ml, to <5% of the uninhibited yield (rows 6 and 7 in Table 4).

DISCUSSION

The role of thyroid hormones at a cellular level in neoplastic transformation is largely unknown. Data from human epidemiological studies are conflicting, though results in animal studies indicate that hypothyroidism is associated with lowered frequencies of some forms of neoplasia, retardation of growth of transplantable tumors, and suppression of metastatic proliferation (13). Thyroid hormone treatment reverses some of these effects.

To manipulate the cellular abundance of thyroid hormone, the fetal calf serum was treated with an anion exchange resin as described (8). The relative selectivity of this procedure in removing low molecular weight organic anions and reversal of the effects by addition of T3 to the medium justified ascribing the effects to changes in thyroidal hormone concentration (2, 8).

In the present studies, transformation and its modulation was assessed under defined conditions in both HEC and C3H/10T$^1/_2$ cells in culture (5, 14). The results indicate that thyroid hormone plays a key role in chemically induced transformation in both cell systems. The absolute dependence of transformation on T3 was found with the indirect carcinogen B[a]P and with the direct-acting agent MNNG. These results imply that the action of T3 is not limited simply to the conversion of B[a]P to an active intermediate but is expressed at the level of the genomic events mediating the process of transformation.

The action of T3, similar to our findings with radiation, appears to be in the early steps of neoplastic transformation in initiation and fixation of the events rather than in later phases associated with expression and promotion (1, 2, 5, 14).

This is evident from the data in the experiments delineating the time dependence of T3 action. A maximum effect on trans-

Table 4. Effects of T3, rT3, and cycloheximide on B[*a*]P-induced neoplastic transformation in C3H/10T1/2 cells*

Medium				
Pretreatment	12 hr before B[*a*]P	48 hr after B[*a*]P	Transformed foci per surviving cells	Transformation frequency
−T3	−T3†	−T3	0/15,000	—
−T3	−T3	−T3	0/12,680	—
+T3	+T3	+T3	14/17,872	7.83×10^{-4}
−T3	+T3	−T3	9/11,680	7.71×10^{-4}
−T3	+rT3	+rT3	0/19,815	—
−T3	+T3‡	+T3	3/25,425	1.18×10^{-4}
−T3	+T3§	+T3	1/26,300	3.80×10^{-5}

*T3 was at 1 nM when present, rT3 was at 1 nM, and B[*a*]P was added at 1.2 μg/ml for a period of 48 hr.
†No B[*a*]P treatment.
‡Cycloheximide at 50 ng/ml was added 12 hr before addition of B[*a*]P and was removed 60 hr thereafter (48 hr after B[*a*]P treatment).
§Cycloheximide at 100 ng/ml was added 12 hr before addition of B[*a*]P and was removed 60 hr thereafter (48 hr after B[*a*]P treatment).

formation rate was exerted when T3 was added to the medium 12 hr prior to B[*a*]P treatment. T3 was less effective in modulating transformation when added simultaneously with B[*a*]P and was ineffective if added 48 hr after B[*a*]P, despite the continued presence of T3 for the entire 4–6 weeks required for expression of transformation.

The dependence of chemically induced transformation on T3 differs significantly from that seen in viral transformations. In rat embryo fibroblasts, removal of T3 from the medium reduced the yield of adenovirus-induced transformations significantly but did not eliminate the response completely (15). The results indicated that T3 exerted its effects both on initiation (i.e., within 72 hr after infection with adenovirus) and on optimal growth of the transformed colonies. A significant reduction in transformation under hypothyroid condition and a dependence on T3 have been obtained recently with transformation induced by the Kirsten virus in rat kidney cells (ref. 16; J. Rhim, personal communication).

The results indicate that T3 is crucial in early phases of chemically induced transformation and has no effect on the later stage of expression. The mode of action in rendering the cells competent for transformation is unknown. However, our present results and earlier findings with radiation (1, 2) imply that T3 may exert its influence by inducing a host protein that plays a key role in the events triggering transformation by physical and chemical agents. Support of this inference includes the following evidence. (*i*) The time dependence of the effect of T3 on transformation is similar to that observed in the induction of host proteins (2, 9–11, 17). (*ii*) The action of T3 on transformation is suppressed by cycloheximide as reported here (Table 4) and elsewhere (2). (*iii*) There is a striking similarity between the concentration-dependent effects of T3 in radiogenic and chemically induced transformation with that of induced Na^+,K^+-ATPase activity (Fig. 1). (*iv*) rT3 [an isomer that has no effect on protein synthesis (11)] does not elicit transformation in response to either x-rays or B[*a*]P (2).

We thank Mr. Herbert Mason for his excellent assistance. This work was supported by National Cancer Institute Grants CA12536 and CA22376.

1. Guernsey, D. L., Ong, A. & Borek, C. (1980) *Nature (London)* **288**, 591–592.
2. Guernsey, D. L., Borek, C. & Edelman, I. S. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 5708–5711.
3. Borek, C. & Sachs, L. (1966) *Nature (London)* **211**, 276–278.
4. Reznikoff, C. A., Bertram, D. W. & Heidelberger, C. (1973) *Cancer Res.* **33**, 3231–3238.
5. Borek, C. (1982) *Adv. Cancer Res.* **37**, 159–232.
6. Huberman, E., Sachs, L., Yang, S. K. & Gelboin, H. V. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 188–192.
7. Sugimura, T. & Kawachi, T. (1973) *Methods Cancer Res.* **7**, 245–308.
8. Samuels, H. H., Stanley, F. & Casanova, J. (1979) *Endocrinology* **105**, 80–85.
9. Lo, C. S. & Edelman, I. S. (1976) *J. Biol. Chem.* **251**, 7834–7840.
10. Lo, C. S. & Lo, T. N. (1980) *J. Biol. Chem.* **255**, 2131–2136.
11. Oppenheimer, J. H. (1979) *Science* **203**, 971–979.
12. Obrig, T. G., Culp, W. J., McKeehan, W. L. & Hardesty, G. (1971) *J. Biol. Chem.* **26**, 174–181.
13. Guernsey, D. L., Borek, C., Fisher, P. B. & Edelman, I. S. (1983) in *Genes and Proteins in Oncogenesis*, eds. Weinstein, I. B. & Vogel, H. J. (Academic, New York), pp. 197–209.
14. Borek, C. & Sachs, L. (1967) *Proc. Natl. Acad. Sci. USA* **57**, 1522–1527.
15. Fisher, P. B., Guernsey, D. L., Weinstein, I. B. & Edelman, I. S. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 196–200.
16. Borek, C. (1983) in *13th International Cancer Congress. Part B: Biology of Cancer I*, eds. Mirand, E. A., Hutchison, W. B. & Mihich, E. (Liss, New York), pp. 105–125.
17. Tata, J. R. (1970) in *Biochemical Actions of Hormones*, ed. Litwack, G. (Academic, New York), pp. 89–133.