THYROXINE 5'-DEIODINATION BY A RAT MAMMARY ADENOCARCINOMA CELL LINE

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ABSTRACT

The metabolism of thyroxine (T4) to 3,5,3'-triiodothyronine (T3) was investigated in cultured mammary tumour cells derived from the R3230AC rat mammary adenocarcinoma. Standard enzyme assay involves the addition of T4 (10 μ M) in the absence of dithiothreitol to the cell monolayer in culture medium. At the end of a 24-h incubation period, T3 produced in the cells was extracted with ethanol and determined by radioimmunoassay. Production of T3 from T4 by the tumour cells was dependent on the number of cells seeded. Increasing the cell number from 1×10^6 to 4×10^6 resulted in a progressive increase in T3 production. The enzyme reaction was linear for at least 48 h and was enhanced by dithiothreitol (0.5–10 mM), inhibited by T3 (0.3 to 30 nM), but was not affected by propylthiouracil (PTU) (0.001 to 1.0 mM). When the cells were pre-incubated for 16 h with different doses (0.6, 6.0 and 12.0 μ M) of actinomycin D, an inhibitor of RNA synthesis, prior to the addition of T4, the production of T3 was greatly enhanced. These data indicate that the cultured mammary tumour cells possess a PTU-insensitive 5'-deiodinase which catalyzes the 5'-deiodination of T4. Although 'superinduction' of the 5'-deiodinase activity can be brought about by actinomycin D, the mechanism by which the enzyme is 'superinduced' by the antibiotic remains to be elucidated.

Biologically active 3,5,3'-triiodothyronine (T3) is generated from thyroxine (T4) by 5'-deiodination; this occurs in a variety of tissues. There are two enzymes, type I and type II, responsible for this reaction (10, 16, 21, 22). The type I enzyme is the propylthiouracil (PTU)-sensitive 5'-deiodinase (5'D-I) which is present in the liver and kidney. The level of this enzyme is decreased in hypothyroidism and stimulated in hyperthyroidism. The deiodination process catalyzed by the type I enzyme provides most of the circulatory T3 in adult animals *e.g.*, in rats (8). The other enzyme, type II, is the PTU-insensitive 5'-deiodinase (5'D-II). It can be found in the pituitary, brain and brown adipose tissues.

The type II enzyme is increased by thyroidectomy, and it provides 50–80% of the intracellular T3 in the pituitary and the brain.

We have previously observed that the R3230AC rat mammary adenocarcinoma (RAC) was capable of metabolising T4 to T3 and rT3 (14). In this study, the 5'-deiodination of T4 was investigated in cultured cells derived from the same R3230AC tumour, the objective being to define some of the properties of the 5'-deiodinase in this tissue.

MATERIALS AND METHODS

Tumour Tissue

The RAC was obtained from the tumour bank

of the Mason Research Institute, Worcester, MA, U.S.A. The tumour line was maintained by subcutaneous dorsal implantation into 50–60-day-old female Fischer 344 rats (Charles River, Quebec) weighing 120–130 g. The animals were kept on Ralston Purina rat chow and tap water *ad libitum*. The tumours were harvested 14–16 days after implantation.

Establishment of the RAC Cell Line

Solid tumour samples, free of necrotic materials and normal surrounding tissues, were washed with ice-cold Hank's buffered saline solution (HBSS) and dissected into small pieces (1–2 mm). The tumour fragments were then pipetted onto 75 cm² culture flask in standard culture medium (RPMI, Roswell Park Memorial Institute; 1,640 containing 100 U penicillin/ml, 100 µg streptomycin/ml, 31 mU insulin/ml, and 2 mM glutamine supplemented with 10% heat-inactivated fetal bovine serum). The explants were incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. The medium was changed daily and after 96 h monolayers of cells were harvested by gentle trypsinization. The cells were then maintained in the standard culture medium. In two consecutive passages, the cultures were cleaned from fibroblasts by limited brief trypsinization and are hence designated as the RAC cell line. Cells were subcultured at weekly intervals after brief trypsinization. This tissue culture adapted cell line maintained stable tumourigenicity, morphology (assessed by histological examination of hematoxylin and eosin stained sections of subcutaneous tumour) and colonization over at least 10 passages.

5'-Deiodinase Assay

The standard enzyme assay involves the seeding of four million confluent cells onto a 100×20 mm plastic Petri dish containing 5 ml standard culture medium. The cells were allowed to grow in this medium overnight at 37°C in a humidified chamber containing 5% CO₂. The cells were then washed free of fetal bovine serum (FBS) and subsequently cultured in FBS-free standard culture medium. After a 60-min preincubation period in this medium, 10 µM T4 was added to the cells.

The enzyme reaction was allowed to proceed, in the absence of dithiothreitol (DTT), for 24 h at 37°C in the presence of 5% CO₂. At the end of the reaction period, cells were harvested by brief trypsinization. T3 produced in the cells was extracted with ethanol. The ethanol extract was dried under a stream of nitrogen. Quantitation of T3 was done by a radioimmunoassay (RIA) procedure using the T3 RIABEAD diagnostic kit (Abbott Lab., North Chicago, IL) after re-dissolving the dried residue in bicarbonate saline buffer provided by the RIA kit. The sensitivity of the assay was 250 pg T3 per ml, and the thyroxine cross-reactivity was 0.1%. Control cultures for each assay sample were set up to correct for any added T3, T3 present in commercial T4 and any cross-reaction of T4 in the T3 RIA. Results were calculated as the difference between the T3 detected in the test and control cultures and were expressed as pmol T3 per 10⁶ viable cells per 24 h. The number of viable cells at the end of the incubation period was generally in the range of 9 to 12×10⁶. The viability of the cells was determined by the trypan blue exclusion method.

RESULTS

Titration of the RAC Cell Numbers for the 5'-Deiodinase Assay

To determine the optimum number of tumour cells to be used for the assay of 5'-deiodinase, T3 production from T4 was determined in cultures plated with tumour cells ranging from 1×10⁶ to 8×10⁶ in numbers. Fig. 1 shows that T3 production from T4 by the RAC cells is dependent on the number of cells used in the assay. Increasing the cell number from 1×10⁶ to 4×10⁶ leads to a progressive increase in T3 production. Maximum T3 production was observed with 4×10⁶ cells. Increasing the cell number to 8×10⁶ did not increase T3 production further.

Time Course of the Production of T3 from T4 by the Cultured RAC Cells

Fig. 2 shows the time course of the 5'-deiodination of T4 by the cultured tumour cells. The production of T3 from T4 was linear for at least 48 h.

Effect of DTT on the 5'-Deiodination of T4 by the Cultured RAC Cells

The effect of a reducing agent on the 5'-deiodination of T4 was studied by using DTT. Fig. 3 shows that 5'-deiodination of T4 was low in the absence of DTT. However, addition of DTT (0.5–10 mM) was observed to enhance progressively the 5'-deiodinase activity of the cultured tumour cells. Despite its potentiating effect, DTT was not included in the standard enzyme assay in this study. This is due to the fact that DTT, even at the lowest concentration (0.5 mM) used, causes the cells to adhere firmly to the plastic Petri dish. Therefore, har-

Fig. 1 Titration of RAC cell numbers for the assay of 5'-deiodinase. Varying numbers (1 to 8 millions) of RAC cells were seeded onto plastic Petri dishes (100 $\times$ 20 mm) in the presence of 5 ml standard culture medium. The cells were allowed to grow in this medium for 24 h in a humidified chamber containing 5% CO₂. Then, the medium was replaced with FBS-free standard culture medium. After a 60-min preincubation period, 10 μ M T4 were added to the cells in DTT-free medium. The enzyme reaction was terminated after 24 h. Cellular production of T3 was determined by radioimmunoassay. Each point represents the mean T3 production $\pm$ SEM of triplicate cultures.

Fig. 2 Time course of the 5'-deiodination of T4 by the cultured RAC cells. Monolayers of cells were incubated with 10 μ M T4, in the absence of DTT, for varying periods of time (0–48 h) in FBS-free standard culture medium at 37°C in a 5% CO₂ humidified atmosphere. Each point represents the mean T3 production $\pm$ SEM of triplicate cultures.

Fig. 3 Effect of DTT on the 5'-deiodinase of cultured RAC cells. Monolayers of RAC cells were cultured with 10 μ M T4 in the presence of varying concentrations of DTT (0.5–10 mM) under standard assay conditions. Each point represents the mean T3 production $\pm$ SEM of triplicate cultures.

Fig. 4 Effect of PTU on the 5'-deiodinase of the cultured RAC cells. Monolayers of RAC cells were cultured in DTT-free medium containing 10 μ M T4 and varying concentrations of PTU (0.001–1.0 mM) under standard conditions. Each point represents the mean T3 production $\pm$ SEM of triplicate cultures.

vesting the cells required repeated scrapping with a rubber policeman, which is a fairly time-consuming step. The addition of DTT, at a concentration starting at 2.5 mM, also markedly reduces the viability of the tumour cells by more than 40%.

Effect of PTU on the 5'-Deiodination of T4 by the Cultured RAC Cells

The effect of PTU, an antithyroid drug, on the production of T3 from T4 by the tumour cell is shown in Fig. 4. It can be seen that at concentrations ranging from 0.001 to 1.0 mM, PTU did not affect the 5'-deiodination of T4 to T3.

Effect of T3 on the 5'-Deiodination of T4 by the Cultured RAC Cells

The effect of substrate analog on the 5'-deiodination of T4 was studied by using T3. In this experiment, the added T3 was subtracted from total T3 detected in the test cultures by inclusion of control cultures containing the same amount of added T3 as the test cultures. Fig. 5 shows that T3 exerted a dose-dependent

Fig. 5 Effect of T3 on the 5'-deiodinase of the cultured RAC cells. Monolayers of cells were cultured in DTT-free medium containing 10 μ M T4 and varying concentrations of T3 (0.3 to 30 nM) under standard assay conditions. The added T3 was subtracted from the total T3 detected in the test cultures by inclusion of control cultures containing the same amount of added T3 as the test cultures. Results are indicated as the difference in T3 detected between the test and control cultures, and are expressed as pmoles T3 per 10⁶ viable cells per 24 h. Each point represents the mean T3 production $\pm$ SEM of triplicate cultures. Statistical analysis (Student's *t*-test) indicated a significant difference between culture (not treated with T3) and cultures treated with 0.3 nM ($P < 0.005$), 1.5 nM ($P < 0.002$), 3.0 nM ($P < 0.002$) or 30 nM ($P < 0.001$) T3.

inhibition of the 5'-deiodination of T4. Increasing the T3 concentrations from 0.3 to 30 nM resulted in a proportionately reduced T3 production from T4. Thus, it appears that the 5'-deiodination of T4 is characterized by product inhibition of the enzyme reaction.

Effect of Actinomycin D on the 5'-Deiodinase in Cultured RAC Cells

Pretreatment of tumour cells with varying doses (0.6, 6.0 and 12.0 μ M) of actinomycin D, an RNA synthesis inhibitor, for 16 h prior to

Fig. 6 Effect of actinomycin D on 5'-deiodination of T4 by cultured RAC cells. Cells were pretreated with actinomycin D (0.6, 6.0 and 12.0 μ M) or without (0 μ M) actinomycin D for 16 h in FBS-free standard culture medium prior to the addition of 10 μ M T4. The enzyme reaction was allowed to proceed in the absence of DTT for 24 h, in a humidified chamber containing 5% CO₂ and 95% air. Each point represents the mean T3 production $\pm$ SEM of triplicate cultures. Statistical analysis (Student's *t*-test) indicates a significant difference between control culture (0 μ M actinomycin D) and cultures treated with 0.6 μ M ($P < 0.00005$), 6.0 μ M ($P < 0.002$) or 12.0 μ M ($P < 0.00005$) actinomycin D.

the addition of T4 resulted in enhanced T3 production (Fig. 6). These data indicate that the 5'-deiodinase in the tumour cells can be 'superinduced' by actinomycin D.

DISCUSSION

The results of this study demonstrate that the tissue-culture-adapted R3230AC rat mammary adenocarcinoma cell line is capable of converting T4 to T3 by the process of 5'-deiodination. This provides direct evidence that the cell line retains the 5'-deiodinase previously observed in the solid RAC tumour (14).

The 5'-deiodinase activity is dependent on the number of cells seeded and the time

course of culture (Figs. 1 and 2). When a thiol-reducing agent, DTT (0.5–10 mM), was included in the assay system, it was observed that the 5'-deiodinase activity in the tumour cells can be enhanced progressively with increasing concentrations of the reducing agent (Fig. 3). These results indicate that like the 5'-deiodinase in the rat liver (3), kidneys (11), pituitary (4, 21), brain (5) and the cultured monkey hepatocarcinoma cell homogenate (17), the 5'-deiodinase of the cultured RAC cell is sensitive to DTT activation.

The mechanism by which DTT activates the 5'-deiodinase in the cultured RAC cells is not known at present. However, it is conceivable that DTT may increase the binding of T4 to its receptor on the cultured tumour cells, either by increasing the receptor affinity or by increasing the number of high affinity binding sites for T4. Alternatively, DTT may exert a protective effect on the sulphydryl groups which may be present at the active site of the enzyme.

In the present experiments, PTU in concentrations ranging from 0.001 to 1.0 mM did not affect the 5'-deiodination of T4 by the cultured RAC cells (Fig. 4). Hence, like the PTU-insensitive 5'-deiodinase of the CNS (22), brown adipose tissues (10, 15) and the pituitary (21), the enzyme in the tumour cells may be a type II 5'-deiodinase.

The 5'-deiodinase of the cultured RAC cells is under feedback inhibition by the end product (T3) of the reaction which it catalyzes. This is evident from the results of the experiments depicted in Fig. 5, in which the 5'-deiodination of T4 in the tumour cells is proportionately reduced in the presence of increasing concentrations (0.3 to 30 nM) of T3. This inhibition by T3 of the 5'-deiodination of T4 in the tumour cells (Fig. 5) lends support to the observations made by Leonard *et al.* (12) and Maeda and Ingbar (13) that the elevated cerebrocortical and pituitary type II 5'-deiodinase levels seen in hypothyroidism are decreased by 70–80% within hours after injection of a saturating dose of T3.

It can be seen from Fig. 6 that pretreatment of the cultured tumour cells for 16 h with actinomycin D (0.6, 6.0 and 12.0 μ M), prior to the addition of substrate, resulted in a greatly enhanced 5'-deiodinase activity. These results indicate that actinomycin D can 'superinduce'

the 5'-deiodinase in the cultured tumour cells.

Superinduction of various enzymes by actinomycin D (2, 7, 19) has previously been reported and several hypotheses have been proposed to explain this phenomenon. According to Tomkins and his colleagues (20), the paradoxical stimulatory effect of actinomycin D could be attributed to the elimination of a repressor of mRNA synthesis. More recently, it has been proposed that actinomycin D may facilitate the translation of specific mRNA (6, 9) or retard the production of degradative enzyme (1, 18). While the mechanism of the superinduction of the 5'-deiodinase in the cultured RAC cells by actinomycin D has yet to be established, it is likely that the antibiotic may exert its 5'-deiodinase superinducing effect by one or more of the mechanisms indicated above.

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