# Induction of Peroxidase and Thyroglobulin by TSH in Cultured Thyroid Cells from Patients with Basedow's Disease and Its Inhibition by Actinomycin D

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**The effects of TSH on peroxidase activity (PO) and thyroglobulin (TG) production were investigated using primary cultures of thyroid cells obtained from patients with Basedow's disease (Basedow's cells). PO activity of cultured cells and TG concentration in the culture medium were measured by biochemical and sandwich enzyme immunoassays, respectively. The addition of TSH (10 mU/ml/day) to the medium did not increase the cell number but did increase the PO activity and TG concentration. It took more than 3 days for the PO activity of cells cultured with TSH (stimulated group) to reach a level twice that of cells cultured without TSH (control group),whereas 2 days of incubation with TSH was sufficient for increasing the TG concentration. When actinomycin D (AD) was added to the medium on the first day of 3-day incubation with TSH, the stimulatory effect of TSH on PO was completely blocked and the TG concentration was reduced to half that of the control group. AD given to the stimulated group on the last day of induction produced no inhibitory effect on the induction of PO activity by TSH, but reduced the TG concentration to almost half that in the stimulated group. An electron microscopic study of Basedow's cells cultured with AD and TSH failed to reveal any cytopathic change. The findings of the present study suggested that in cultured Basedow's cells TSH induces PO activity and TG production through the synthesis of new messenger RNA. Acta Pathol Jpn 39: 121-126, 1989.**

**Key words:** Thyroid culture, Peroxidase, Thyroglobulin, TSH, Actinomycin D

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## INTRODUCTION

TSH stimulates the secretion of thyroid hormone by modifying the synthesis of various proteins (1).

Using thyroid glands of experimental animals, many authors have reported that the activity of thyroid peroxidase (PO), a key enzyme for thyroid hormone synthesis, is also controlled by TSH (2-6). However, no study has yet reported that TSH actually increases the PO activity of human thyroid cells. Moreover, although TSH is considered to stimulate PO activity through synthesis of new protein (3), the question of whether TSH stimulates this protein synthesis at the transcriptional or translational level is still unsettled.

In this study, using cultured thyroid cells obtained from patients with Basedow's disease, we found that TSH increased both peroxidase activity and the production of thyroglobulin (TG), and stimulated the synthesis of both proteins at the transcriptional level.

## MATERIALS AND METHODS

### 1. Cell culture

Human thyroid cells obtained from patients with Basedow's disease (Basedow's cells) were cultured with minimal essential medium (Flow Labs. Inc., U.K.) supplemented with penicillin 50U/ml (Gibco, U.S.A), streptomycin 0.05 mg/ml (Gibco) and 10% fetal bovine serum (M.A. Bioproducts., U.S.A.) at 37°C in a humidified incubator, in an atmosphere of 5% $CO_2$, as reported previously (7). In brief, surgically removed thyroid tissue was minced with scissors and dispersed with a magnetic stirrer in culture medium containing collagenase 10 mg/dl (Boehringer, West Germany), deoxyribonuclease 2 mg/dl (Boehringer), and dispase 1,000 U/ml (Goudousyusei, Japan) for 45 min at 37°C. Then $1 \times 10^6$ cells were incubated in a 3-cm plastic dish (Nunc, Denmark)

with 2 ml of the culture medium, which was changed every day.

Most cells obtained by the above method showed positive immunoreaction for thyroglobulin on the second day of the culture (7). According to the experimental schedules described in RESULTS, TSH (Whale, Sigma) and actinomycin D (Sigma) were added to the culture medium. Actinomycin D, which had been dissolved in 100% ethyl alcohol, and TSH were diluted with the culture medium. As the Basedow's cells obtained from one patient were limited in number (about $10\times10^6$ per gland), the samples used in this study were different in individual experiments. A total of 22 thyroid glands were used.

## 2. Assay methods for peroxidase (PO) and thyroglobulin (TG)

PO activity was measured as reported previously (7) with minor modification. In brief, the cultured cells were frozen with 1 ml distilled water at −80°C. For assay of PO activity, cells were incubated at 37°C with an additional 1 ml of citric acid buffer solution (0.1 M., pH = 4.8) containing o-phenylenediamine (OPD) and hydrogen peroxide ($H_2O_2$). The final concentrations of OPD and $H_2O_2$ were 1 mg/ml and 0.02%, respectively. After incubation for 30 min, 1 ml 6 N sulfuric acid was added to stop the enzyme reaction. Then the optical density of the buffer solution colorized by endogenous peroxidase was read at 405 nm (OD 405).

TG in the culture medium was measured by sandwich enzyme immunoassay as reported previously (7). In brief, 50 μl TG-containing culture medium was transferred to a well of a 96-well microplate to which anti-TG antibody had been bound. After incubation for 1 h and washing with Tween 20, a second anti-TG antibody labelled with horseradish peroxidase was added and incubated for an additional 1 h. Then after washing, the activity of horseradish peroxidase was assayed using a method identical to that for endogenous peroxdiase described above. The amounts of citric acid buffer, OPD and sulfuric acid added per well were 100 μl, 0.8 mg/ml and 50 μl, respectively. The amount of TG in the culture medium (ng/ml) was calculated by comparison of the OD values of the medium to those of standard samples.

## 3. Measurement of uridine uptake by cultured Basedow's cells

Cultured cells were incubated with actinomycin D (2.5, 5 and 10 μg) and [$^3$H] uridine (5 μCi), and with or without TSH. After incubation for 5 h and 24 h, respectively, the cells were washed once with phosphate-buffered saline without calcium and magnesium, and lysed with Triton X-100 (1% in distilled water). Then 10 μl of this solution was spotted onto DE-81 paper. The paper was placed in a vial containing scintillation fluid and the uptake of [$^3$H] uridine was counted with a liquid scintillation counter.

## 4. Ultrastructural study

Electron microscopy was performed as reported previously (7) to study the effects of actinomycin D on cultured Basedow's cells. In brief, cultured cells were fixed with 50% Karnovsky's fluid for 30 min, and incubated in 0.05 M Tris-HCl buffer (pH 7.6) containing 3, 3′-diaminobenzidine-tetrahydrochloride (0.5 mg/ml) and hydrogen peroxide (0.0025%) for 1 h. After osmication with 1% osmium tetroxide for 30 min and dehydration in a graded ethanol series up to 100%, the cells were embedded by inverting a gelatin capsule of Epon 812 mixture over the cells. After polymerization of Epon 812 mixture, the cells attached to the bottom of the block were removed from the dish by dissolving the dish with xylene.

# RESULTS

The chronological changes in the numbers of Basedow's cells cultured with or without TSH (stimulated and control groups, respectively) are shown in Table 1. Among 15 comparisons, the number of cells in the control group was larger than that in the stimulated group on 8 occasions and the two were almost the same on 3 occasions. The probability of the occurrence of this result was 0.19 (calculated by sign test). The ratio of cell number between the stimulated and control groups in each comparison was around 1 and did not exceed 1.5. Therefore, we concluded that TSH does not

**Table 1.** Number of Basedow's Cells ($\times10^5$) Cultured with or without TSH

| Case | TSH (10 mU/ml) | Incubation days after addition of TSH | | |
|---|---|---|---|---|
| | | 2 | 4 | 6 |
| 1 | + | 1.7[a] | 1.7 | 1.6 |
| | − | 1.7 | 1.6 | 1.7 |
| 2 | + | 4.5 | 4.1 | 2.9 |
| | − | 3.2 | 3.9 | 3.5 |
| 3 | + | 3.6 | 3.7 | 3.4 |
| | − | 3.7 | 3.9 | 2.9 |
| 4 | + | 2.7 | 2.4 | ND |
| | − | 3.1 | 2.9 | ND |
| 5 | + | 3.6 | 1.5 | ND |
| | − | 3.6 | 2.2 | ND |
| 6 | + | 1.4 | 1.4 | ND |
| | − | 1.6 | 1.4 | ND |

[a] Figures below the fifth digit were rounded.
From cases 1 to 3, +: 10 mU/ml TSH was added daily to the medium after preparation of Basedow's cells from thyroid and from cases 4 to 6, +: Basedow's cells were cultured for 3 days without TSH and then 10 mU/ml TSH was given daily.
ND: not done.

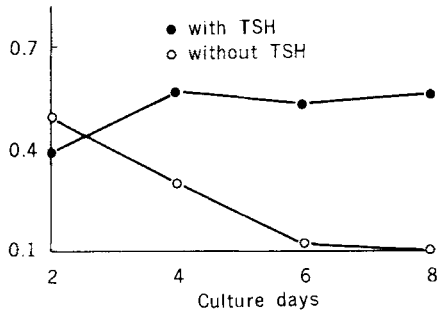


**Figure 1.** Chronological change in O.D. values for PO activity of Basedow's cells cultured with or without TSH (10 mU/ml).

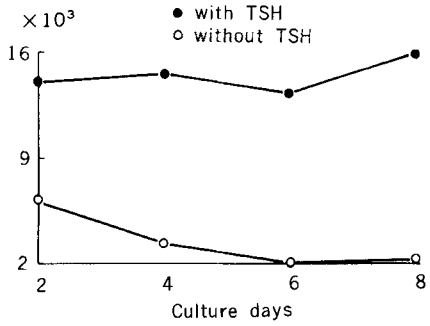


**Figure 2.** Chronological change in TG concentration (ng/ml) in culture medium after addition of TSH.

stimulate the growth of Basedow's cells under the present culture conditions.

The chronological changes in PO activity and TG concentration of Basedow's cells from one patient are shown in Figs. 1 and 2. PO activity and TG concentration were measured after incubation with or without 10 mU/ml TSH daily for 2, 4, 6 and 8 days. No difference of PO activity was observed between the stimulated and control groups until after 2 days of incubation, although after 4 days of incubation, the PO activity in the stimulated group became double that in the control group, and then reached a plateau that lasted until the end of the experiment. The TG concentration in the stimulated group was double that in the control group shortly after 2 days of incubation.

**Table 2.** Relative Values for PO Activity and TG Concentration after incubation with 10 mU TSH

| Group | Incubation with or without TSH for 3 days | PO activity | TG concentration |
|---|---|---|---|
| 1 | − − − | 100[a] | 100[a] |
| 2 | − − + | 110±21 | 148±59 |
| 3 | − + + | 147±37 | 264±85 |
| 4 | + + + | 231±36 | 232±67 |

Number of samples in each group was 5.
+ : TSH (10 mU/ml/day) was added to the culture medium.
[a] Relative value (RV) of PO activity or TG concentration was calculated as follows; $RV = V(n)/V(1) \times 100$
V(n): O.D. values for endogenous peroxidase activity of cultured Basedow's cells or TG concentration in culture medium (ng/ml) in group n.
± : standard deviation.

The relationship between shorter incubation period with TSH and induction of PO activity and TG concentration is shown in Table 2. As values of PO activity and TG concentration were different from case to case, they were expressed as ratios to those of group 1 (control group). It took 3 days of incubation with TSH (group 4) for PO activity to reach a level double that in group 1, and the probability of occurrence of the values of PO activity between groups 1 and 4 calculated by sign test was 0.031. The TG concentration in the stimulated group reached a level twice that of the control group soon after 2 days of incubation.

Even when Basedow's cells were cultured for 3 days without TSH and then TSH was added daily to the culture medium, it also took 3 and 2 days of incubation with TSH in order to increase the PO activity and TG concentration, respectively (data not shown). In order to observe the inductive effect of TSH on PO and TG, Basedow's cells cultured without TSH for 3 days were used in the following experiments.

The inhibitory effects of actinomycin D (AD) on the increase of PO activity and TG concentration by TSH are shown in Table 3. When 2.5 $\mu$g/ml AD was added to the medium on the first day of 3-day incubation with

**Table 3.** Inhibitory Effect of Actinomycin D (AD) on Induction of PO Activity and TG Concentration by TSH

| Group | Treatment for 3 days | | Relative Value of PO Activity | Relative value of TG Concentration |
|---|---|---|---|---|
| 1 | TSH | − − − | 100[a] | 100 |
| | AD | − − − | | |
| 2 | TSH | + + + | 100±18 | 4±2 |
| | AD | + − − | | |
| 3 | TSH | + + + | 147±37 | 51±23 |
| | AD | − + − | | |
| 4 | TSH | + + + | 231±36 | 352±56 |
| | AD | − − + | | |
| 5 | TSH | + + + | 244±48 | 564±109 |
| | AD | − − − | | |

Number of samples in each group was 5.
[a] Relative value (RV) was calculated as RV in Table 2.
+ : TSH (10 mU/ml/day) or AD was added to the medium.

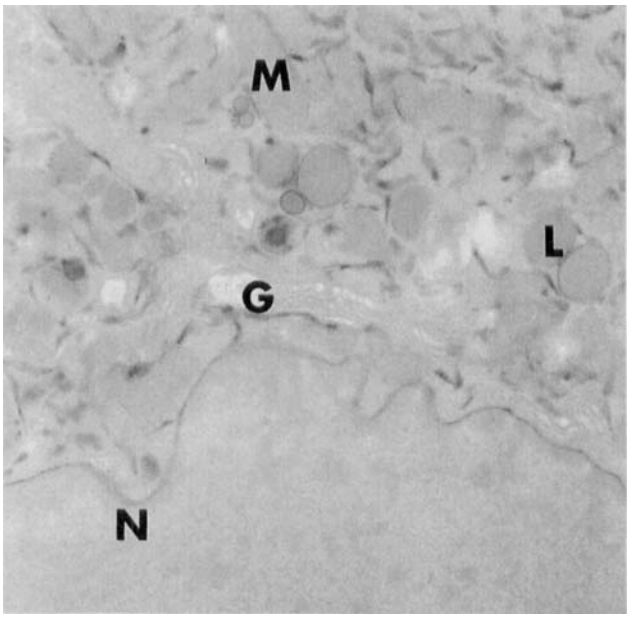


**Figure 3.** Ultrastructural appearance of a Basedow's cell in the control group after cytochemical reaction for PO activity. PO activity is observed mainly in the perinuclear space and cisternae of rough endoplasmic reticulum. N, nucleus; G, Golgi complex; L, lysosome; M, mitochondrion. ×10,000. Stained with lead citrate for 3 min.

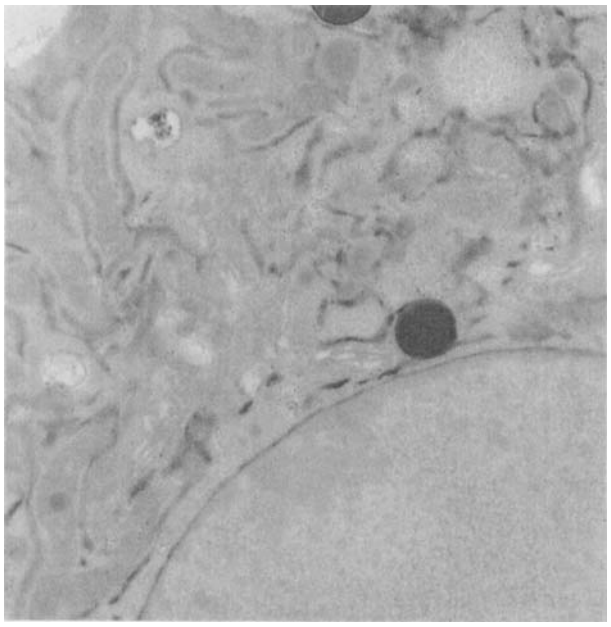

**Figure 4.** Ultrastructural appearance of a Basedow's cell in the inhibitory group for the same sample as that in Fig. 3 after cytochemical reaction for PO activity. No difference of localization of PO activity or distribution of cell organelles is evident between the control and inhibitory groups. ×10,000. Stained with lead citrate for 3 min.

TSH (inhibitory group), no increase of PO activity was observed, whereas when AD was added on the last day of the incubation, no inhibitory effect of AD was observed. In the case of TG, the inhibitory effect of AD was observed even when AD was added on the last day of the incubation, although the effect became much smaller than that when AD was added on the first day of the incubation. Addition of 2.5 $\mu$g/ml AD to the culture medium inhibited uridine uptake by Basedow's cells (Table 4).

**Table 4.** Inhibitory Effect of Actinomycin D (AD) on [$^3$H] Uridine Uptake by Cultured Basedow's Cells (cpm)

| Incubation Period | Amount of AD Added to Culture Medium ($\mu$g/ml) | | | |
|---|---|---|---|---|
| | 0 | 2.5 | 5.0 | 10 |
| 5 h without TSH | 3000[a)] | 270 | 100 | 60 |
| 24 h with TSH[b)] | 40000 | 110 | 93 | ND |
| 24 h without TSH | 60000 | ND | 145 | 90 |

Only one case was used in this study.
[a)] Counts of beta-rays from [$^3$H] uridine incorporated into cultured cells.
[b)] The amount of TSH was 10 mU/ml.
ND: not done.

The ultrastructural localization of peroxidase activity in Basedow's cells from the control group (cultured without TSH for 6 days) obtained from a patient (Case No. 13) is shown in Fig. 3. Among the 5 thyroid glands studied ultrastructurally, peroxidase activity was preserved best in this sample and was localized mainly in perinuclear cisternae and cisternae of rough endoplasmic reticulum. The ultrastructural localization of peroxidase activity in Basedow's cells of the inhibitory group for the same gland is shown in Fig. 4. Basedow's cells of the inhibitory group were cultured without TSH for 3 days, with TSH (10 mU/ml) and actinomycin D (2.5 $\mu$g/ml) for 1 day, and with TSH only for 2 days. There was almost no difference in the localization of peroxidase and the distribution of subcellular organelles between the control and inhibitory groups.

## DISCUSSION

The present study showed that TSH did not stimulate the growth of thyroid cells obtained from patients with Basedow's disease. This result seems to contradict the reports that TSH has tumorigenic effects on the thyroid gland (8, 9). In *in vitro* studies, however, the roles of TSH in the growth of normal thyroid cells have not yet been settled (10-16); moreover, it has been reported that TSH did not stimulate the growth of thyroid cells obtained from cases of goiter (13) and from goitrogen-treated thyroid gland (14). Therefore, we concluded that TSH does not stimulate the growth of thyroid cells from cases of toxic diffuse goiter, at least under the present culture conditions.

The present study revealed that TSH increases the intracellular PO activity of Basedow's cells and the concentration of TG released by the cells into the culture medium. Although we did not assay the amount of intracellular TG, we considered that TSH stimulated the production of TG because no decrease in cell number was

noted during the period of the experiment, thus denying the possibility that the increased TG came from dead cells, and the amount of TG in the culture medium roughly correlates with the total amount of intracellular TG (Noguchi S, personal communication). Moreover, addition of TSH to the medium in which Basedow's cells had been cultured for more than a week without TSH increased the level of thyroglobulin in the medium (unpublished data).

As the induction of PO activity by TSH requires a certain times period, from 1 to 7 days (2-6), it has been considered that TSH induces PO activity through *de novo* protein synthesis, and Nagasaka and Hidaka reported that cycloheximide, an inhibitor of protein synthesis at the translational level, prevented TSH-induced restoration of thyroid peroxidase activity in hypophysectomized rats (3). Magnusson and Rapoport reported that not only TSH but also dibutyl cAMP, 8-bromo-cAMP, forskolin, and cholera toxin, all of which are known to increase the level of intracellular cAMP, induced PO activity in cultured dog thyroid cells, and stated that induction of peroxidase by TSH is a cAMP-mediated phenomenon (6). In their study, the question of whether this cAMP-mediated signal of TSH stimulates the transcriptional level or translational level of peroxidase synthesis was not investigated because of the toxicity of actinomycin D (AD). They stated that canine thyroid cells were unable to tolerate incubation with AD for 1 day (6). However, Ishii *et al.* reported that cultured thyroid cells obtained from patients with hyperthyroidism were able to tolerate 5-day incubation in a medium containing 10 $\mu$g/ml AD added daily (17). Therefore, we used thyroid cells from cases of toxic diffuse goiter for the present study.

We found that addition of 2.5 $\mu$g/ml AD to the medium showed a marked inhibitory effect on the uridine uptake of Basedow's cells cultured with or without TSH. In contrast, no cytopathic effect of AD on the cells cultured with TSH was observed ultrastructurally. Therefore, we consider that the inhibitory effect of AD on the induction by TSH observed in the present study was due not to its cell toxicity but to its inhibitory effect on RNA synthesis. As the stimulatory effect of TSH on PO and TG was blocked by the addition of AD, we consider that TSH stimulates the synthesis of PO and TG at the transcriptional level.

The present study showed that the interval between the addition of TSH and AD and the appearance of the drugs' effects on PO activity differed from the case of TG concentration. Whether this difference is due to differences in the synthesis of PO and TG at the transcriptional, translational or post-translational processes including the process of TG release remains to be studied.

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