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Functional Expression of Thyrotropin Receptor in Differentiated 3T3-L1 Cells: A Possible Model Cell Line of Extrathyroidal Expression of Thyrotropin Receptor

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Thyrotropin receptor (TSHR) in extrathyroidal tissue, especially fat tissue, is supposed to have important roles in the development of extrathyroidal manifestations of Graves' disease. However, the molecular mechanism of TSHR expression is not known. Expression of TSHR mRNA and TSH-dependent cAMP production were observed in differentiated but not in undifferentiated 3T3-L1 cells. Maximal expression was obtained when the cells were differentiated in the presence of insulin, dexamethasone, and isobutylmethylxanthine (IBMX). Dexamethasone and IBMX were indispensable for the first three days. On the other hand, after day 4, insulin was indispensable for the expression of TSHR. 3T3-L1 cell is the first non-thyroidal cell line reported that expresses TSHR and whose expression can be induced. 3T3-L1 cell can be a good model to investigate the mechanism of expression of TSHR and extrathyroidal manifestations of Graves' disease.

Autoimmune reactions are involved in the pathogenesis of thyroidal and extrathyroidal manifestations of Graves' disease. Thyroid stimulating antibody (TSAb) binds to thyrotropin receptor (TSHR) in human thyroid gland and causes hyperfunctioning of thyroid gland (1). Patients with Graves' disease who have high titers of anti-TSHR antibodies, are often accompanied by exophthalmos and pretibial dermopathy (2, 3). Therefore, if TSHR in extrathyroidal tissues serves as an antigen common to thyroid gland, this could explain the development of extrathyroidal manifestations of Graves' disease.

Actually, TSH binding activity in fat tissue has long been known (4, 5). Marcus et al. (6, 7) showed that human fat tissues also have TSHR. Recently, we reported that rat epididymal fat tissues are extremely abundant in TSHR mRNA (8). However, the mechanism of extrathyroidal expression of TSHR has not been studied. The expression of thyroid-specific proteins such as thyroglobulin (9), thyroid peroxidase (10), and TSHR (11) are controlled by thyroid-specific transcription factor (TTF-1). Among them, only TSHR exists in fat tissue. In this paper, we report that conversion of undifferentiated 3T3-L1 cells to adipocyte phenotype is accompanied by the expression of functional TSHR, thus providing the means of investigating the basis for expression of TSHR in extrathyroidal tissues.

MATERIALS AND METHODS

Cell culture. A mouse preadipocyte cell line 3T3-L1 (American Type Culture Collection, Rockville, MD) was cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM) containing 10% calf serum and brought into a full differentiation as described by McGehee et al. (12). One day after confluence (day 0), the cells were treated with differentiation medium (DMEM containing 10% fetal bovine serum (FBS), 10 μg/ml insulin, 0.2 μg/ml dexamethasone, and 0.5 mM isobutylmethyl xanthine) for 3 days. After removing the differentiation medium, cells were fed DMEM containing 10% FBS and 10 μg/ml insulin every other day. At the end of the culturing, the cells in 12-well plates were

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stained with oil-red O and hematoxylin as described by Gaillard et al.(13). Pictures of the cells and an internal ruler (one scale = 10 μm) were taken under a microscope, after which the sizes of the fat droplets were measured.

Northern blot analysis. Total RNA was isolated from cells by the guanidine isothiocyanate extraction method (14). 20 μg of total RNA was electrophoresed in 1% formaldehyde-agarose gel and blotted onto a nitrocellulose filter as described previously (8). A mouse thyroid TSHR cDNA was PCR-amplified by using mouse thyroid TSHR cDNA specific probes (15) (5′-primer:5′-TCTGAGTACTACAACCACGC, 3′-primer:5′-TGAAGAAGCCAGC-CGTGTTG) and used as the probe for Northern blot analysis. Direct sequencing of the PCR amplified cDNA from 3T3-L1 cells verified the TSHR mRNA. Rat β-actin cDNA, kindly donated by Dr. L. D. Kohn (NIH, Bethesda, MD), was labeled with [α-^{32}P]-dCTP by using a random primer labeling kit (Takara Shuzo, Co., Kyoto, Japan). Blots were hybridized in 50% formamide, 2.5× Denhardt's solution, 5× SSPE (0.6 M NaCl, 40 mM sodium phosphate, 4 mM EDTA, pH 7.4), 0.1% SDS, 0.1 mg/ml heat-denatured salmon sperm DNA, and 5% dextran sulfate. Hybridization was performed at 42°C for 12 h with radio-labeled probes. The filters were then washed three times at room temperature in 2× SSC (0.3 M NaCl, 30 mM sodium acetate, pH 7.0) containing 0.1% SDS, followed by washing three times at 53°C in 0.1×SSC containing 0.1% SDS. The filters were exposed to an imaging plate and analyzed using Bas 2000 image analyzer (Fuji, Film Co, Tokyo, Japan).

cAMP measurement. Cells in 24-well culture plates (Corning, Iwaki Glass Co., Tokyo) were washed once with the cAMP assay buffer (DMEM containing 10 mM Hepes, pH 7.4, and 1 mM IBMX) and preincubated in the buffer for 30 min. The cells were then incubated with additives in 0.5 ml of cAMP assay buffer in the presence of 2 U/ml adenosine deaminase for 30 min at 37°C. cAMP was measured (16) by using a commercially available RIA kit (Yamasa, Choshi, Japan). Bovine TSH was obtained from Sigma (MA, USA).

Glycerol measurement. Cells were grown in 12-well plates and washed once with the glycerol assay buffer (DMEM without phenol and pyruvate containing 10 mM Hepes, pH 7.4, 1% bovine serum albumin (Fraction V) (Sigma, MA. USA), and 1 mM IBMX) and preincubated with the buffer for 30 min at 37°C. The cells were, then incubated with the additives in 0.3 ml of glycerol assay buffer for the indicated period of time. After the incubation, the medium was collected and heated at 65°C for 10 min. The glycerol released into the medium was measured using the glycerol assay kit, "Glycerol-F" (Boehringer Mannheim, Mannheim, FDR).

RESULTS

TSHR mRNA was not detected in undifferentiated 3T3-L1 cells (day 0) and not until the fifth day following the initiation of differentiation. However, by day 7, TSHR mRNA became evident, reaching maximum, as revealed by Northern blot analysis (Fig. 1A), at day 11. The changes in the amount of TSHR mRNA coincide with morphological differentiation of the cells (Table 1) as judged by the percentage of the cells which had lipid droplets. At day 7, about 90% of the cells contained lipid droplets, which are the characteristic feature of mature fat cells. The sizes of TSHR

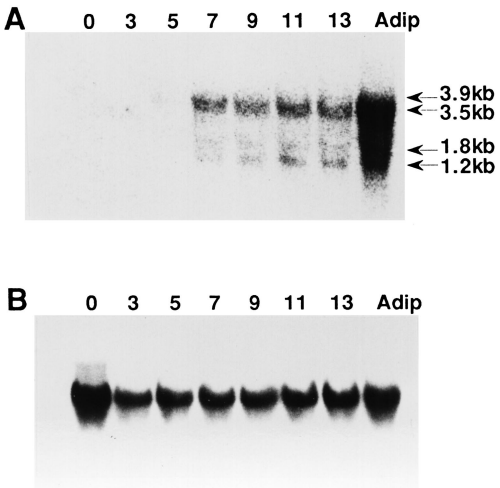


FIG. 1. Expression of TSHR mRNA (A) and β actin mRNA in 3T3-L1 cells. Northern blot analysis of total RNA (20 μg/ml) from 3T3-L1 cells before (day 0) and after the differentiation. ^{32}P-Labeled mouse thyroid TSHR cDNA (A) and rat β actin cDNA (B) were used as the probes. The numbers above each lane denote the days after the initiation of differentiation. kb:kilobase.

TABLE 1
Oil-Red O Staining of 3T3-L1 Cells

	Size of fat droplets		
Days	0	~10 μm	10 μm≤
	ratio of the cells (%)		
day 1	100	0	0
day 3	36.4	57.4	6.2
day 7	5.9	89.1	5.0
day 11	5.0	59.5	35.5

The cells were cultured in 12-well plates under usual differentiation condition for the days indicated in the table and stained with oil-red O and hematoxylin. 300 cells from each well were evaluated and classified according to the sizes of the lipid droplets. The data are representative of two identical experiments.

mRNA bands of 3T3-L1 were same as those of mouse thyroid (data not shown) and epididymal adipocytes. The amount of β actin mRNA was largest in undifferentiated 3T3-L1 cells (day 0), but remained similar during the course of the differentiation (days 3–13) (Fig. 1B).

TSH-dependent production of cAMP was not observed in undifferentiated cells (days 1, 3, and 5). On day 7, TSH stimulated the production of cAMP. The stimulation was maximal at day 11 (Fig. 2). The concentration of TSH needed to cause cAMP production was 1 mU/ml. When adenosine deaminase was excluded from the buffer to see an inhibitory effect of endogenous adenosine, production of cAMP was inhibited. Recombinant human TSH also stimulated cAMP production by differentiated 3T3-L1 cells (data not shown).

An experiment was designed to know a good condition during the initiation of differentiation (from day 1 to day 3) to induce the expression of TSHR mRNA (Fig. 3A) and cellular differen-

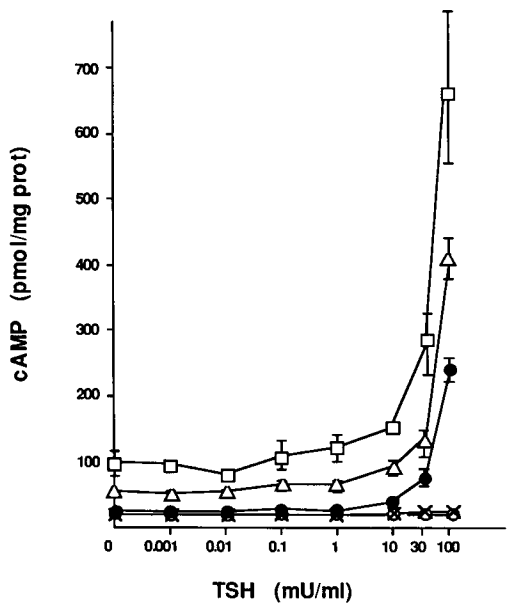
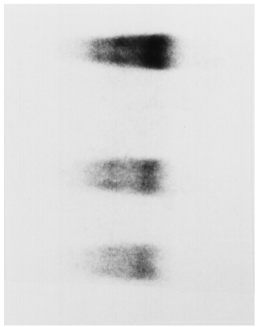


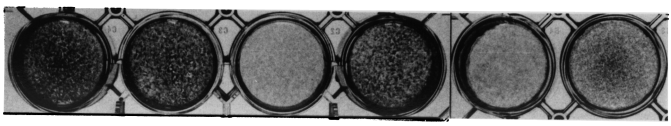
FIG. 2. Differentiation-dependent cAMP response of cultured 3T3-L1 cells to TSH. One day after confluency, 3T3-L1 cells were incubated in differentiation medium for 3 days, then in DMEM containing 10% FBS and 10 μg/ml insulin. The cells were incubated with increasing concentrations of bovine TSH in cAMP assay buffer for 30 min. Total cAMP was measured. The cells were from the day 3, ×; day 5, (◯); day 7, (●); day 9, (△) and day 11, (□). Values are the mean ± SEM (n = 3).

A



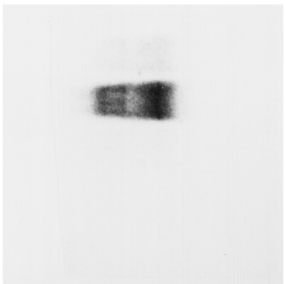
	Culture Conditions	
	Day 1~3	Day 4~10
1	IDX	I
2	ID	I
3	IX	I
4	DX	I
5	I	I
6	IDX	I → none*

B



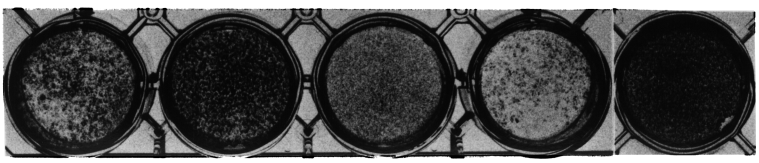
1 2 3 4 5 6

C



	Culture Conditions	
	Day 1~3	Day 4~10
1	IDX	none
2	IDX	I
3	IDX	X
4	IDX	D
5	IDX	IDX

D



1 2 3 4 5

FIG. 3. Effects of culture conditions on the expression of TSHR mRNA and morphological differentiation. Culture conditions were changed during the first three days (A, B) or afterwards (days 4–10) (C, D). A, C; TSHR mRNA was detected using Northern blot analysis of total RNA (20 μg/lane) from 3T3-L1 cells. ^{32}P-Labeled mouse thyroid TSHR cDNA was used as a probe. Autoradiograph was put laterally so that the upside of the gel comes on the right side. Cells were cultured in DMEM containing 10% FCS with or without I, insulin (10 μg/ml); D, dexamethasone (0.2 μg/ml); X, IBMX (0.5 mM). B, D; Cells were cultured in 12-well plate and stained with oil-red O. Picture of the plate was shown. The numbers of each well in B and D correspond to the culture conditions described in A and C, respectively. *Cells were cultured in the presence of insulin on day 4 and day 5. Then the cells were deprived of insulin.

tiation (Fig. 3B). Dexamethasone and IBMX were indispensable from day 1 to day 3. When IBMX was omitted during the time, cellular differentiation was obtained, but the expression of TSHR mRNA was not (Fig. 3A-2). Without dexamethasone, cellular differentiation and expression of TSHR mRNA were not obtained (Figs. 3A-3, 3B-3).

In other experiments, after the first three days of differentiation with insulin, dexamethasone, and

IBMX, cells were cultured from day 4 to day 10 under different conditions. The cells cultured with only insulin after day 4 were well differentiated and were most abundant in TSHR mRNA (Fig. 3C-2). Although the cells cultured in the presence of insulin, dexamethasone, and IBMX for 10 days were most abundant in fat droplets (Fig. 3D-5), surprisingly, they expressed no TSHR (Fig. 3C-5). Dexamethasone and IBMX added singly after day 4 did not stimulate differentiation (Fig. 3D-3, 4) or the expression of TSHR (Fig. 3C-3, 4).

Glycerol released into the medium was measured to relate TSH-stimulated cyclic AMP production with lipolysis (Table 2). 1 mU/ml of TSH was needed to cause lipolysis in 1 h; 0.1 mU/ml of TSH in 6 h.

DISCUSSION

Thyroid gland is a highly differentiated organ which expresses thyroid-specific proteins such as thyroglobulin (9), thyroid peroxidase (10), and TSHR (11). Many evidences (17–19) support the importance of TTF-1 and TTF-1 binding elements as the most important determinants of thyroid-specificity. In marked contrast, rat adipose tissue and 3T3-L1 cells do not have TTF-1, yet is abundant in TSHR mRNA. These findings infer that TSHRs in 3T3-L1 cells and rat fat cells have escaped from the usual network of transcription factors responsible for expression of thyroid-specific proteins.

Investigating the mechanism of extrathyroidal expression of TSHR using 3T3-L1 cells could lead to the better understanding of extrathyroidal manifestations of Graves' disease. The existence of a common antigen between thyroid and retro-orbital tissue is important in the development of Graves' ophthalmopathy. If TSHR is the common antigen, this may explain why severe exophthalmos is often found in patients with Graves' disease who have strong anti-TSHR antibodies. Many studies concerning the mechanism of targeting of the T cell-mediated autoimmunity within the retro-orbital tissues (20, 21) have been done using fibroblasts obtained from retro-orbital tissues. On the other hand, despite the fact that orbital fat tissue was enlarged in Graves' exophthalmos (22, 23), detailed biochemical studies were not done with orbital fat cells. The model system of 3T3-L1 cells reported here should facilitate such investigations.

In the present study, 3T3-L1 cells were fully differentiated in the presence of insulin, dexamethasone, and IBMX. The levels of morphological differentiation during the course of culturing compared favorably with the expression of TSHR mRNA. Thus, TSHR can be classified as one of the proteins whose expression are induced in differentiated 3T3-L1 cells (24–26). Among the additives tested, insulin is known to increase TSHR mRNA in FRTL-5 cells through an insulin responsive element (IRE). As for the effect of IBMX, it is odd that IBMX added from day 1 to day 3 increased TSHR mRNA if it is assumed that IBMX leads to increased cAMP: theoretically this should decrease TSHR mRNA (27). Actually, IBMX added after day 4 did not stimulate cellular differentiation and the expression of TSHR mRNA. Dexamethasone had similar effects. Therefore, the effects of IBMX and dexamethasone seem to depend on the stage of cellular differentiation. In any case, if we presume direct actions of insulin, dexamethasone, or IBMX on TSHR gene, it is difficult to explain why only fat cells but not other cells are rich in TSHR mRNA. We suggest

TABLE 2
TSH-Stimulated Glycerol Production

Incubation time	TSH 0.1	1	10	30 (mU/ml)
1h	0.11 ± 0.36	2.29 ± 0.27	4.20 ± 0.09	5.20 ± 0.04 (μg/well)
6h	2.31 ± 0.85	10.14 ± 0.47	17.39 ± 3.6	21.72 ± 1.51 (μg/well)

Fully differentiated 3T3-L1 cells (day 11) were used. Basal glycerol production obtained in the absence of TSH was subtracted.

intervening factor(s), specific to adipocytes, which enables expression of TSHR under the control of the hormones. Possible contributions of transcription factors which play major roles in adipogenesis (28, 29) should be elucidated in future studies.

In conclusion, TSHR is expressed in 3T3-L1 cells in a differentiation-dependent manner. 3T3-L1 cell line is so far the only and the best cell line that enables us to study the mechanism of expression of TSHR in extrathyroidal tissues.

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