Immunohistochemical localization of thyroid hormone nuclear receptors in human hair follicles and in vitro effect of L-triiodothyronine on cultured cells of hair follicles and skin

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Summary : To investigate the cellular basis of the action of thyroid hormone on hair follicles, we studied the immunohistochemical localization of thyroid hormone receptors (TRs) in human scalp skin using a mouse monoclonal antibody, TRα1 (C4) against TRs. Immunoreactive TRs were detected in the nuclei of the outer root sheath cells (ORSCs), dermal papilla cells (DPCs), fibrous sheath cells of hair follicles, hair arrector pili muscle cells and sebaceous gland cells. However, nuclei of hair matrix cells were not clearly stained with TRα1 (C4). The epidermis showed positive nuclear staining by the antibody. Ductal and secretory portions of eccrine sweat glands were also stained with the antibody as we had expected. In the dermis, almost all the cell components including fibroblasts, vascular endothelial and smooth muscle cells, and Schwann cells were positively stained. Immunofluorescence also showed TRs expression in cultured ORSCs, DPCs, epidermal keratinocytes and dermal fibroblasts. L-triiodothyronine stimulated the proliferation and / or metabolism of all these four types of cells significantly, although there was variation at the rate of stimulation. Whereas, structurally similar, but metabolically inactive analog, reverse T_3 had no effect. These results demonstrate the presence of thyroid hormone nuclear receptors in human hair follicles. Furthermore, the presence of TRs in different cell types in the skin suggests numerous direct effects of thyroid hormone on this target tissue. J. Med. Invest. 44 : 179-184, 1998

IITRODUCTION

Thyroid hormone (T_3) regulates growth, differentiation, and development of a variety of tissues. Hypothyroidism leads to dry, coarse, and brittle hairs that become increasingly thinner. Sparse scalp hair, loss of the outer third of the eyebrows, and diminished body hair are often seen in hypothyroidism (1). The relative proportions of telogen compared to anagen hairs in the scalp increase in patients with hair loss due to hypothyroidism, suggesting either prolonged telogen or premature catagen or both (2). This abnormality is corrected by the administration of thyroid hormone. Again in hyperthyroidism, scalp hair tends to be fine and soft. Altered texture and diffuse alopecia are also seen in hyperthyroidism (1). These observations indicate that the hormone has effects on both the hair cycle and growth of human hair. However, the mechanism by which the hormone regulates hair growth has not been elucidated.

There is general agreement that most of the biological effects of thyroid hormones are mediated by an interaction of L-triiodothyronine (L-T_3) with thyroid hormone nuclear receptors (TRs) (3, 4). Therefore, the presence of TRs is of critical importance for the effects of thyroid hormones on hair follicles. Although the presence of TRs in human skin has been demonstrated by reverse transcription-polymerase chain reaction (RT-PCR) (5), little information is available about the expression of the receptors in hair follicles. In the present investigation, we have immunohistochemically examined TRs expression in human scalp skin, especially in hair follicles, and studied the exogenous effect of L-T_3 on the proliferation of different types of cultured cells obtained from hair follicles and skin in vitro.

MATERIALS AND METHODS

Specimens and cells

Skin samples were taken from seven patients without systemic disease undergoing dermatologic surgery at Tokushima University Hospital, Tokushima, Japan. All cell strains used in this study were primary cells and initiated in this laboratory as previously described (6, 7, 8). Dermal papilla cells (DPCs) and dermal fibroblasts (DFs) were cultured in Dulbecco's modified Eagle's medium

(DMEM) supplemented with 12% fetal calf serum, if not mentioned otherwise. Outer root sheath cells (ORSCs) and epidermal keratinocytes (EKs) were cultured in keratinocyte growth medium, K-GM (Kurabo Biomedical Business, Osaka, Japan), if not otherwise specified. Cells of 2 to 4 passages were used in this study.

Immunohistochemistry

The expression of TRs was immunohistochemically examined using a mouse monoclonal antibody, TRα1 (C 4) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), raised against a peptide mapping within the carboxy terminus of the human thyroid receptor α1. Seven scalp skin specimens obtained from different individuals were fixed in 10% neutralized formaldehyde and processed routinely to paraffin-embedded sections. After deparaffinization, tissue sections were treated with microwave for retrieval of an antigen as previously described (9). Sections were soaked in 10% milk for 10 min to block nonspecific binding of antibodies and then incubated with TRα1 (C4) at a concentration of $5 \mu g/ml$ for 90 min at room temperature in a humidified chamber. After washing with phosphate-buffered saline (PBS ; 10 mM sodium phosphate, pH 7.5 and 0.15 M NaCl), they were treated successively with affinity-purified goat antibody against mouse IgG ($5 \mu g / ml$) and avidin-biotin-peroxidase complexes of a Vectastain ABC kit (Vector Lab., Burlingame, CA, USA) as recommended by the manufacturer. Bound peroxidase complexes were visualized using a solution of 0.01% 3,3-diaminobenzidine tetrahydrochloride, 10 mM imidazole, and 0.01% hydrogen peroxide in 0.1 M Tris-HCl (pH 7.2). As negative controls for the immunostaining, normal mouse IgG ($5 \mu g/ml$) instead of TRα1 (C 4) and as positive controls for the immunostaining, human kidney obtained at autopsy, and rat kidney and pituitary gland taken from a male Sprague-Dawley rat were used (10, 11).

Immunofluorescence

Four types of cells (DPC, DF, ORSC, and EK) were plated at a density of 1 to 3×10^3 cells/cm^2 onto Lab-Tek chamber glass slides (Nunc Inc., Naperville, IL, USA) and cultured for 48 h. After washing with PBS, cells on the slides were fixed in 10% neutralized formaldehyde for 30 min at room temperature. Following microwave treatment, cells were treated with 0.05% Triton X-100 in PBS for 5 min and then washed with PBS three times. Cells were incubated with TRα1 (C4) ($5 \mu g/ml$) for 90 min at room temperature. After washing with PBS, fluorescein isothiocyanate-conjugated F(ab')2 fragments of rabbit immunoglobulins to mouse immunoglobulins (Dakopatts A/S, Glostrup, Denmark) were applied at 1 : 20 dilution for 60 min at room temperature. The slides were then washed in PBS, mounted with Perma Fluor (Lipshaw/ Immunon, Pittsburg, PA, USA), and observed through a Nikon fluorescence microscope (Nikon Instech Co., Tokyo, Japan). As negative controls for the immunostaining, normal mouse IgG ($5 \mu g/ml$) instead of TRα1 (C4) was used. Two strains were examined in each cell type.

L-T$_3$ bioassay on the cultured cells

Cells were seeded at a density of 5×10^3 cells/well into Sumilon 96-well plastic plates (Sumitomo Bakelite Co., Tokyo, Japan) and cultured for 24 h in the media described above. Then, the culture media were changed to DMEM containing 1% fetal calf serum for DPCs and DFs, and a mixture of MCDB-153 medium (Sigma Chemical Co., St. Louis, MO, USA) and K-GM at 9 : 1 ratio for ORSCs and EKs. Both media were supplemented with or without L-T$_3$ (Sigma Chemical Co.) at concentrations ranging from 2×10^{11} M to 2×10^7 M. Metabolically inactive hormone reverse T$_3$ (rT$_3$) (Sigma Chemical Co.) was used at a concentration of 2×10^9 M. Both L-T$_3$ and rT$_3$ were first dissolved in dimethylsulfoxide and then added in the medium (final concentrations of vehicle ≤ 0.05%) as described by Hanley et al (12). Cells were cultured for 3 days in the test medium and then evaluated for cell proliferation by a colorimetric method (WST-1 assay) (8, 13). Eight wells of a Sumilon plate were prepared for each concentration. Three strains of each cell type were used and experiments were conducted at least three times with each strain. Statistical analyses were carried out using Student's t-test, and $P < 0.05$ was considered to be significant.

RESULTS

Tissues from kidney and pituitary gland had previously been demonstrated to express nuclear TRs (10, 11). To confirm sensitivity and specificity of TRα1 (C4) in immunohistochemical analysis, therefore, we examined paraffin sections of human and rat kidneys, and rat pituitary gland for reactivities of the antibody with nuclei. Initial experiments using sections untreated with microwave resulted in failure to detect nuclear staining in the tissues. However, when sections were treated with microwave, the antibody clearly reacted with nuclei of the tissues tested (Fig. 1 A) compared with the negative controls (Fig. 1 B), although cytoplasmic staining was occasionally observed. This indicated that TRα1 (C4) could be useful for immunohistochemical detection of TRs.

In scalp skin specimens, TRα1 (C4) clearly reacted with nuclei of epidermal keratinocytes in all layers up to stratum granulosum (Fig. 1 C). In hair follicles, nuclear TRs were detected in dermal papilla cells in the hair bulb (Fig. 2). In hair matrix cells, we failed to detect nuclear staining although cytoplasmic staining was observed (Fig. 2). The nuclei of ORSCs were strongly stained (Fig. 3) and those of fibrous sheath cells were also positively stained with the antibody (Fig. 3). Staining intensity of DPCs appeared to be slightly weaker than that of ORSCs, although quantitative analysis was not performed. In the dermal area, immunoreactive TRs were located in the nuclei of fibroblasts, hair arrector smooth muscle cells, sebaceous gland cells (Fig. 4), and ductal and secretory portions of eccrine sweat glands (Fig. 3). TRs were also found in both the endothelial and smooth muscle cells of blood vessels and Schwann cells (Fig. 5).
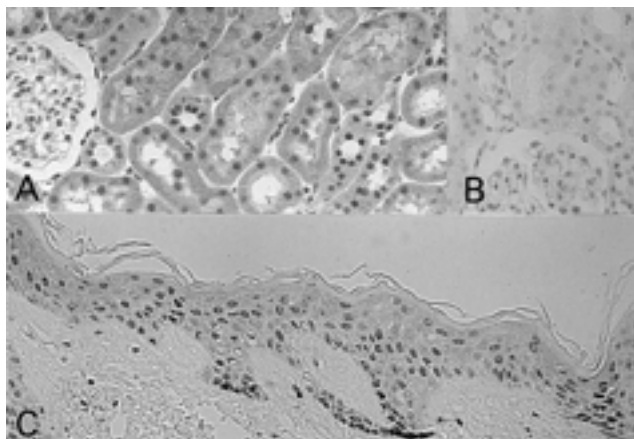
Fig.1. Immunohistochemical staining of rat kidney tissue (A), epidermis of scalp skin (C) using a monoclonal antibody TRα1 (C4), and negative control of rat kidney tissue (B) using normal mouse IgG instead of TRα1 (C4). Paraffin-embedded sections were immunohistochemically stained as described in Materials and Methods. TRα1 (C4) reacted with nuclei of the rat kidney tubules. In the epidermis, intense nuclear staining was found in all layers up to stratum granulosum (x 60).
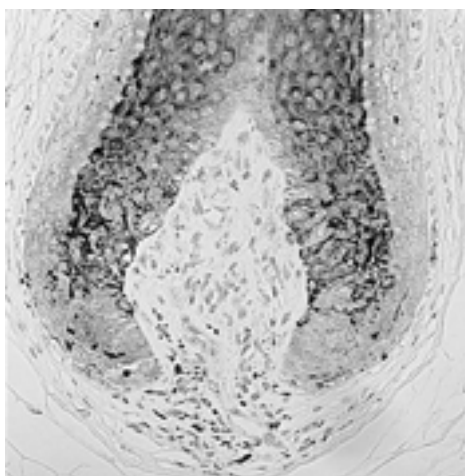

Fig.2. Immunohistochemical detection of TRs in dermal papilla cells in the hair bulb. Nuclear staining was not detected in hair matrix cells, although cytoplasmic staining was observed. Dark pigment in the matrix represents melanin granules (x 120).

We also examined TRs expression in cultured ORSCs, DPCs, EKs, and DFs by indirect immunofluorescence using TRα1 (C4). All these cells showed clear nuclear fluorescence with the antibody (Fig.6), but none with normal mouse IgG (data not shown).

We next examined the effect of L-T$_3$ on the proliferation of ORSCs, DPCs, EKs, and DFs. L-T$_3$ at concentrations of 2×10^{10} M and 2×10^{9} M significantly stimulated the proliferation of all four types of cells (Fig.7). On the other hand, an inactive form of triiodothyronine, rT$_3$, had no proliferative effect on any type of cells at a concentration of 2×10^{9} M (Fig.7). The effect of L-T$_3$ was also found at a lower concentration of 2×10^{11} M in DFs and EKs. However, DPCs and ORSCs showed little effect on proliferation at the lower concentration of 2×10^{11} M.

DISCUSSION

There is clinical evidence that thyroid hormones have effects on hair growthh (1). However, the mechanism by which the hormones exert the effects on hairs is still poorly understood. In the present study, we immunohistochemically demonstrated the presence of TRs in the cells related with hair follicles such as ORSCs, DPCs, fibrous sheath cells, arrector pili muscle cell and sebaceous gland cells, which provides evidence that these cells are target cells
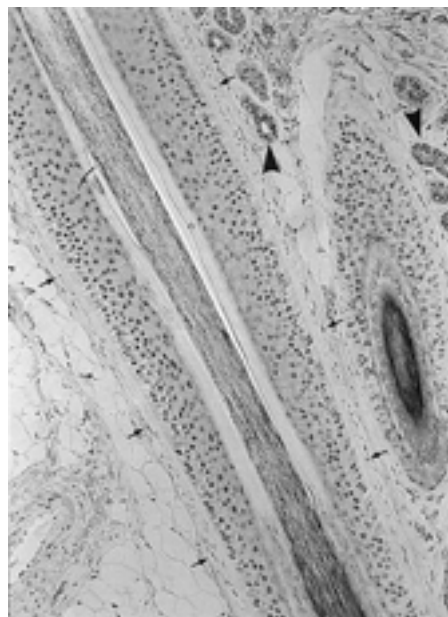

Fig.3. Immunohistochemical localization of TRs in human hair follicles. TRs were found in ORSCs, fibrous sheath cells (arrows), and ductal and secretory cells of eccrine sweat glands (arrowheads) (x 30).
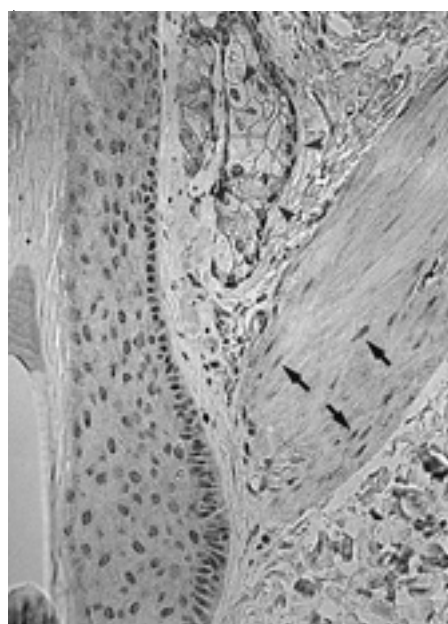

Fig.4. Higher magnification of a hair follicle around the bulge area. Immunoreactivity was detected in the nuclei of hair arrector smooth muscle cells (arrows) and sebaceous gland cells (arrowheads) (x 60).
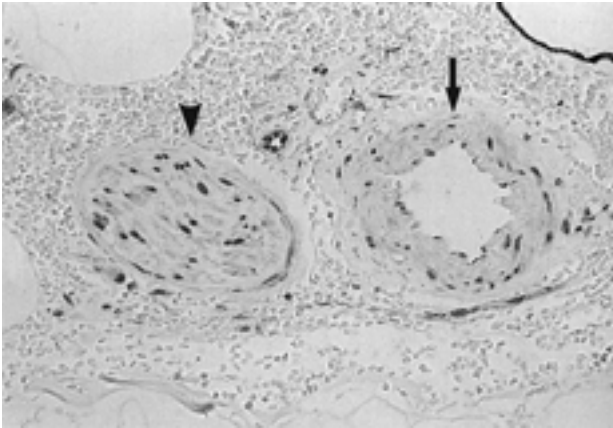
Fig.5. TR immunoreactivity was also found in endothelial and smooth muscle cells of blood vessels (arrow) and schwann cells (arrowhead) (x30).
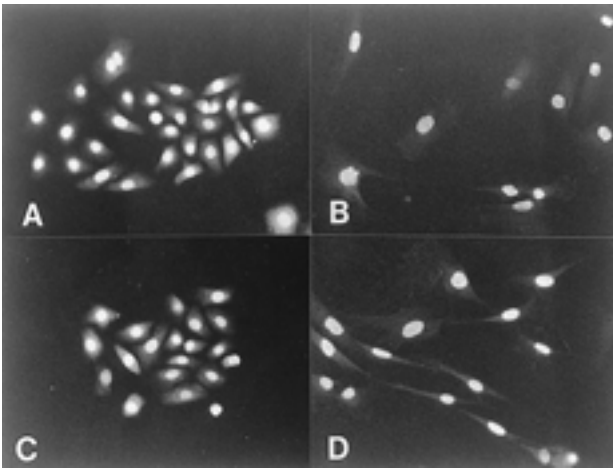

Fig.6. Immunofluorescent staining of TRs in cultured cells. Nuclear fluorescence was detected in all four types of cells examined: (A) ORSCs, (B) DPCs, (C) EKs, (D) DFs (x60, FITC).
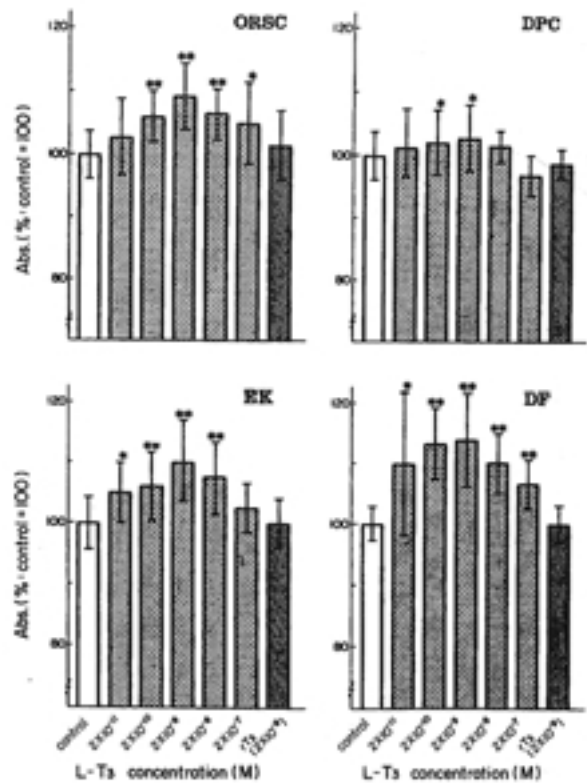

Fig.7. Effect of L-T3 on cell proliferation. ORSCs, DPCs, EKs, and DFs were cultured for 3 days in the test medium supplemented with different concentrations of hormone, as described in Materials and Methods. The results are expressed as percentage of controls. The values are mean ± SD of eight wells. Similar results were obtained in three separate experiments with three separate strains in each case. *P<0.05, **P<0.01 vs. the respective control.

for thyroid hormone.

We also showed the presence of TRs in cultured ORSCs, DPCs, EKs and DFs, and then confirmed significant effect of exogenous L-T$_3$ on the proliferation of all four types of cells at a physiological concentration in vitro, although there was variation at the rate of proliferation among the types of cells used. These data indicate the presence of functional TRs in cultured ORSCs and DPCs. Thyroid hormone, therefore, might have a direct effect on the growth of hair follicles via its receptors.

Two distinct but related genes of TRs, which encode TRa and TRβ, have been identified so far and each gene produces multiple isoforms of TR by alternative splicing (14). The amino acid sequences in the carboxy terminal of TR show significant homology between TRα1 and TRβ1 (14). Although TRα1 (C4), used in the present study was raised against a peptide within the carboxy terminus of human TRα1 isoform, little information was available about the cross reaction of the antibody with other isoforms. Since the presence of TRα1 and TRβ1 isoforms in human skin and cultured keratinocytes were previously shown by RT-PCR (5), nuclear staining with the antibody

in scalp skin and cultured cells in the present study might suggest the presence of both TRα1 and TRβ1 isoforms. However, which isoform(s) of TR is/are expressed in hair follicles remains to be determined.

Freinkel et al. (2) observed failure or delay in the resumption of anagen in hypothyroid patients leading to an increase in the number of telogen hairs and demonstrated that hair changes in hypothyroidism were due to suppression of hair growth. Moreover, in hyperthyroidism hairs become fine and soft. In this study, we found that the growth of all cell strains tested was stimulated by L-T$_3$ dose-dependently up to 2×10^9 M, after which a dose-dependent decrease in proliferation was observed. The maximum stimulation was observed at a serum concentration of 2×10^9 M. DPCs responded to L-T3 at a lesser extent than ORSCs in vitro, which appears to be due to the different amount of TRs expressed in each cell type (11). In this study we evaluated for cell proliferation using a colorimetric method (WST-1 assay). It should be noted that the WST-1 signal is directly correlated with the capacity of mitochondrial enzymes of viable cells to convert WST-1 tetrazolium salt into WST-1 formazan (13). So there is a possibility that the increase of the WST-1 signal is due to the stimulated mitochondrial enzymatic activity by L-T$_3$. However, because other studies using similar colorimetric method such as MTT assay have shown the close correlation between the MTT signal and cell numbers (15) or [^{3}H] thymidine incorporation in

the cells (16), the WST-1 signal seen here may reflect the cell number and metabolic activity. Thus it can be concluded that L-T₃, at concentrations lower or higher than the serum level is not appropriate to promote the proliferation and/or metabolism of hair follicle cells, and this can be directly correlated with the changes of hair seen in both hypo- and hyperthyroidism. Our observations is consistent with previous report that the epidermal proliferation is affected by triiodothyronine (17). However, not all patients with hypo- and hyperthyroidism show hair changes. It is likely that the magnitude of the effect of thyroid hormone on hair growth is variable and its expression may be influenced by local factors and/or other hormones (2, 18).

On the other hand, no nuclear TRs were detected immunohistochemically in hair matrix cells, though the cytoplasms were stained. A possible reason of the failure to detect TRs in matrix cells could be that hair matrix cells actually lack TRs. If so, growth effects of thyroid hormone might be mediated through DPCs, because it has been demonstrated using the co-culture system that DPCs secrete soluble mitogenic substances for epithelial cells (19). This type of action pathway was proposed and confirmed in the case of androgenic action in androgen-dependent hair follicles such as beard and mustache (20). Interestingly, in the hair follicles, androgen receptors were not detected immunohistochemically in the hair matrix cells though ORSCs showed clear expression (21, 22). We also had difficulties in judging nuclear staining of hair matrix cells because of the presence of large amount of melanin pigment and of the dense staining (cross reaction) of cytoplasm. Furthermore, culture of pure hair matrix cells has not been possible so far, therefore, we can not confirm the existence of TRs in cultured cells in vitro. Immunohistochemically TRs were also detected both in sebaceous gland cells, and duct as well as secretory cells of sweat glands. Altered secretion of sweat and sebaceous glands has previously been reported both in hypo- and hyperthyroidism (23, 24). Our findings confirm that thyroid hormone has certain effects on both the glands via TRs.

In conclusion, using a immunohistochemical method, we have demonstrated TRs distribution in human hair follicle as well as in human skin. Through these receptors, thyroid hormone can exert a direct effect on hair follicles. The presence of TRs in a variety of cell types in the skin reflects the multiple effects of thyroid hormone on the skin, although the expression of TRs somewhat differed in different types of cells.

ACKNOWLEDGMENTS

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