Induction of follicle formation in long-term cultured normal human thyroid cells treated with thyrotropin stimulates iodide uptake but not sodium/iodide symporter messenger RNA and protein expression

T Kogai[1], F Curcio[2], S Hyman[3], E M Cornford[3,4], G A Brent[1] and J M Hershman[1]

[1]Endocrinology Division, West Los Angeles VA Medical Center and UCLA School of Medicine, Los Angeles, California 90073, USA

[2]Dipartimento di Patologia e Medicina Sperimentale e Clinica, Università degli Studi di Udine, Udine, Italy

[3]Department of Neurology, UCLA School of Medicine, Los Angeles, California 90095, USA

[4]Southwestern Regional VA Epilepsy Center, Research and Neurology Services, West Los Angeles VA Medical Center, Los Angeles, California 90073, USA

(Requests for offprints should be addressed to J M Hershman, West Los Angeles VA Medical Center, Endocrinology and Metabolism Division 111D, 11301 Wilshire Boulevard, Los Angeles, California 90073, USA; Email: jhershmn@ucla.edu)

Abstract

Iodide uptake by the sodium/iodide symporter (NIS) in thyrocytes is essential for thyroid hormone production. Reduced NIS activity has been reported in thyroid diseases, including thyroid cancer and congenital hypothyroidism. The study of iodide uptake in thyrocytes has been limited by the availability of appropriate *in vitro* models. A new culture technique was recently developed that allows normal human thyroid primary culture cells to grow as monolayer cells and express differentiated functions for more than 3 months. We used this technique to study the effect of follicle formation and TSH on iodide uptake in these cells. Iodide uptake by the cells grown in monolayer was very low. Follicle formation was induced from monolayer cells, and electron micrographs demonstrated cell polarity in the follicles. No significant increase in iodide uptake was observed after TSH treatment of cells in monolayer or when follicle formation was induced without TSH. TSH stimulation of follicles, however, significantly increased iodide uptake (~4·4-fold; $P<0·001$). Compared with iodide uptake in monolayers, the combination of follicle formation and TSH treatment stimulated iodide uptake synergistically to 12·0-fold ($P<0·001$). NIS messenger RNA (mRNA) and protein levels were almost the same in both monolayer cells and follicles. TSH treatment of monolayers and follicles produced significant ($P<0·05$) stimulation of mRNA (~4·8- and ~4·3-fold respectively) and protein (~6·8- and 4·9-fold respectively). TSH stimulated NIS protein levels in both monolayer and follicles, however, stimulation of functional iodide uptake was only seen with TSH stimulation of follicles. The function of NIS may involve post-transcriptional events, such as intracellular sorting, membrane localization of NIS or another NIS regulatory factor. Polarized functions, such as iodide efflux into follicular lumina, may also contribute to the increased iodide concentration after follicle formation.

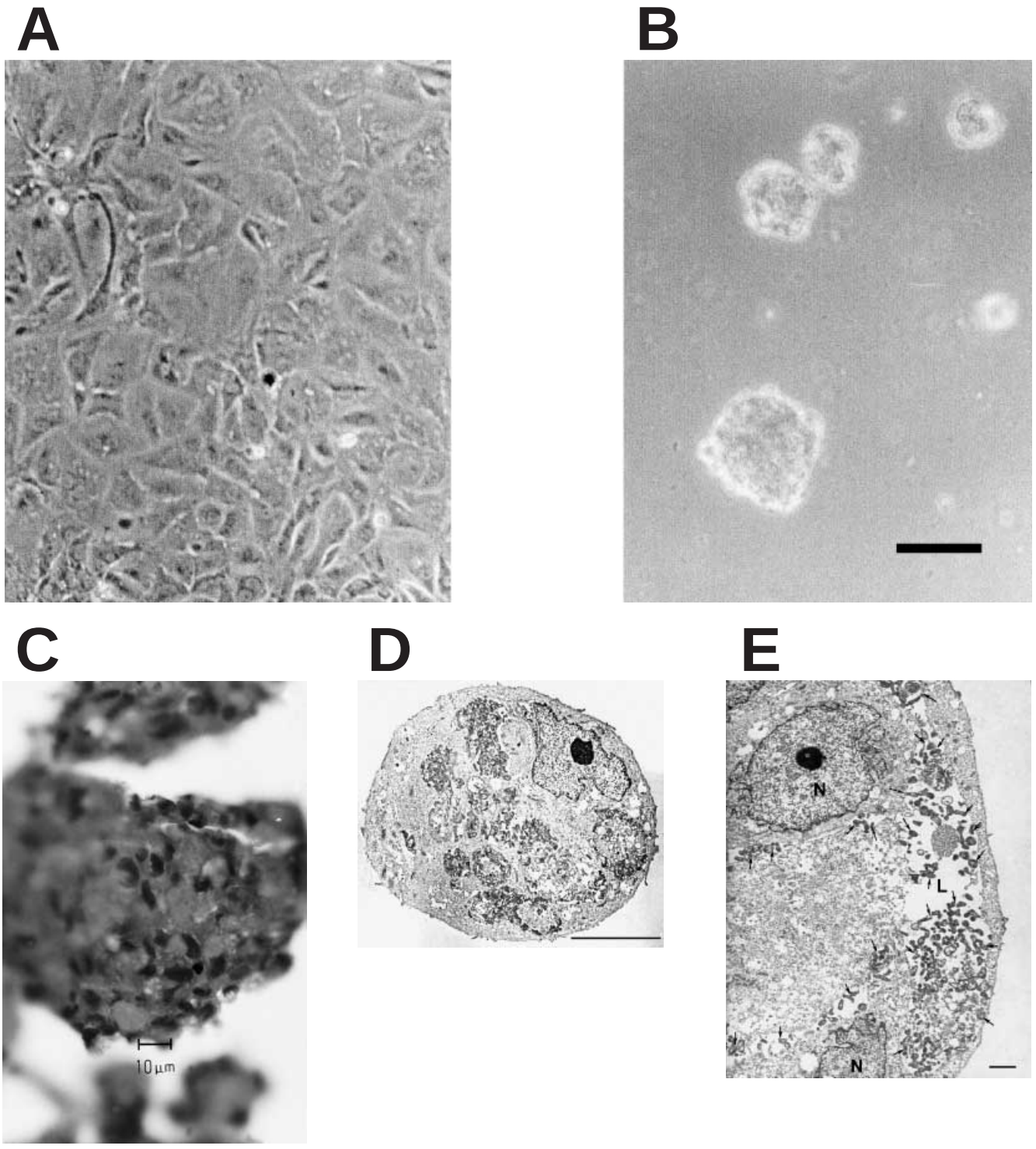
Journal of Endocrinology (2000) **167,** 125–135

Introduction

The transport of iodide into thyroid cells is essential for the synthesis of thyroid hormone (Taurog 1986). This process is catalyzed by the sodium/iodide symporter (NIS) on the thyrocyte plasma membrane (O'Neill *et al.* 1987, Carrasco 1993, Dai *et al.* 1996) and stimulated by thyroid-stimulating hormone (TSH) via the cAMP–adenylate cyclase pathway (Halmi *et al.* 1959, Wilson *et al.* 1968, Knopp *et al.* 1970, Weiss *et al.* 1984). Impaired NIS activity is seen in some thyroid cancers (Saito *et al.* 1998) and rarely in congenital hypothyroidism (Fujiwara *et al.* 1997, Pohlenz *et al.* 1998, Kosugi *et al.* 1998). Studies with FRTL-5 cells, a rat functional thyroid follicular cell line, and with human primary thyroid culture cells reveal that TSH stimulates NIS mRNA and NIS protein expression (Kogai *et al.* 1997, Saito *et al.* 1997, Ajjan *et al.* 1998, Pekary & Hershman 1998). The amount of NIS protein in thyrocytes, however, does not always correlate with measurements of iodide uptake (Kogai *et al.* 1997, Saito *et al.* 1997). Another example of the disparity between NIS expression and iodide uptake occurs in papillary thyroid cancer. NIS mRNA and protein levels are increased in some papillary thyroid carcinomas, although the

0022–0795/00/0167–125

iodide uptake is usually reduced (Saito *et al.* 1998). These studies suggested that NIS activity is regulated not only by NIS gene expression but also by post-transcriptional events, such as translocation or NIS protein turnover, or by other regulatory proteins (Kaminsky *et al.* 1994, Kogai *et al.* 1997, Saito *et al.* 1998).



As there are no functional human thyroid cell lines that can be maintained in long-term culture, studies of NIS expression and activity have been limited to the FRTL-5 cell line or to human thyroid cells cultured for 7–10 days. FRTL-5 cells can be cultured easily (Ambesi-Impiombato *et al.* 1980); however, they do not exhibit cell polarity and NIS protein is randomly distributed on their plasma membrane (Paire *et al.* 1997). In studies of rat and human thyroid tissue, NIS is not expressed throughout the cells, but is clustered in the basolateral membrane (Paire *et al.* 1997, Jhiang *et al.* 1998). In addition, previous studies have demonstrated that follicle structure and cell polarity are required for full activity of transcellular iodide transport in human (Dickson *et al.* 1981, Roger *et al.* 1988, Kraiem *et al.* 1991, Eggo *et al.* 1996), porcine (Nakamura *et al.* 1990, Takasu *et al.* 1992, Nasu & Sugawara 1994), and ovine (Becks *et al.* 1992) primary thyroid cultures.

Recently, one of the authors developed a long-term culture method for primary human thyroid cells (Curcio *et al.* 1994). This method allows for cell proliferation for at least 3 months and the expression of differentiated functions such as TSH-dependent cAMP production, thyroglobulin (Tg) production, and expression of thyroperoxidase mRNA (Curcio *et al.* 1994). The cells also express thyroid-specific transcription factor-1 (TTF-1) and Pax-8 (Perrella *et al.* 1997), which are required for organization and differentiation of thyroid cells (Damante & Di Lauro 1994).

In the present report, we evaluate the iodide uptake activity and NIS mRNA and protein levels in long-term cultured human thyroid cells in monolayer conditions and in follicles with and without TSH stimulation.

Materials and Methods

Monolayer cell culture

The basic culture medium composition was that described previously (Curcio *et al.* 1994). Briefly, it consists of a Coon's modified Ham's F-12 medium (Sigma, St Louis, MO, USA) supplemented with 5% fetal calf serum (FCS) (Gibco, Grand Island, NY, USA), bovine insulin (1 μg/ml) (Sigma), bovine transferrin (5 μg/ml) (Sigma), and hydrocortisone (0·01 nM) (Sigma). Freshly frozen bovine hypothalamus and bovine pituitary (Pel Freeze Biologicals, Rogers, AR, USA) extracts were prepared as described previously (Coon *et al.* 1989), and added to final protein concentrations of 75 and 5 μg/ml medium respectively.

Thyroid tissue was obtained from the normal, non-affected lobe in eight patients with papillary carcinoma or follicular adenoma who underwent thyroidectomy. Serum TSH levels of all patients were normal (1·6–3·0 μU/ml). Use of human surgical material was approved by the institutional human subjects protection committee. Cell dissociation procedures, all performed under sterile conditions, were similar to those used previously (Curcio *et al.* 1994). Briefly, normal human thyroid tissue was freed from adherent connective tissue, cut in small (less than 1 mm diameter) pieces, and washed in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (HBSS). The enzymatic digestion was performed for 2 h with a solution consisting of 20 U/ml collagenase (Sigma), 0·75 mg/ml trypsin (Gibco, 1:300), and 2% heat-inactivated dialyzed chicken serum (Gibco) in Ca^{2+}- and Mg^{2+}-free HBSS (CTC) (Coon *et al.* 1989). Cells were seeded at a density of 10^5 per 100 mm dish. Cells were fed with fresh basic culture medium twice a week and passaged with CTC, as described previously (Curcio *et al.* 1994). Some of the cells were frozen with 10% dimethyl sulfoxide and kept in liquid nitrogen until the next experiment as described (Curcio *et al.* 1994). Thyroid specimens from six patients were used for culture. Five of them were utilized for the iodide uptake study and Northern blot or Western blot analysis, or both. The other specimen was used for the iodide organification and electron microscopic studies.

FRTL-5 cells, kindly provided by Dr L D Kohn (NIH, Bethesda, MD, USA), were maintained as previously described (Ambesi-Impiombato *et al.* 1980). They were treated with or without 0·1 mU/ml bovine TSH for 5 days before iodide uptake assay.

Follicle induction

The follicle-induction medium consisted of Coon's modified Ham's F-12 medium supplemented with 0–1% FCS, bovine insulin (1 μg/ml), bovine transferrin (5 μg/ml), hydrocortisone (0·01 nM) with or without 0·1 mU/ml bovine TSH (Sigma). Monolayer cells cultured for 1–2 months were digested with CTC and plated at a density of 10^6 per 100 mm agarose-coated dish and maintained in

Figure 1 Photomicrograph of long-term cultured normal human thyroid cells. (A) Follicular cells in monolayer condition. Cells were maintained in the basic culture medium for 24 days. (B) Follicle-forming cells. Cells were maintained in the complete medium for 34 days and then seeded in agarose-coated dishes. They formed aggregates in the extract-free medium containing 0·1 mU/ml TSH 1 day after seeding. Bar represents 50 μm. (C) PAS-staining of follicles (6 μm section). Bar represents 10 μm. (D) Low magnification (bar represents 10 μm) electron micrograph cross-section of a TSH-treated cluster of follicle-forming cells. Note that the dark-staining nuclei can be recognized, along with the electron-dense microvilli that fill the intracellular lumens. (E) Higher magnification (bar represents 2 μm) electron micrographic view of a portion of an aggregated cluster of follicle-forming cells, demonstrating that the TSH-treated cells exhibit a distinct (basal-apical) polarity. External borders of the aggregated cells form basal regions and the apical cell surfaces are highly amplified, with numerous electron-dense microvilli (indicated by arrows) projecting into the inter-cellular lumen (L). A prominent, dense nucleous may be seen within the follicle cell nucleus (N).

Table 1 Tg production by human primary thyroid cells. Populations of cells that were used in subsequent studies, from three different patients (1, 2 and 3), were tested. Tg was measured in supernatants from non-clonal follicular thyroid cell populations in cultures derived from normal human thyroid specimens. Twenty-four-hour conditioned medium was collected from individual plates and assayed for Tg. These populations were tested sequentially as shown

	Culture period after operation (days)	**Tg** (fg/cell per day)
Cell population		
1	28	261
1	45	234
2	25	114
2	56	111
3	28	168
3	56	190

follicle-induction medium. Agarose-coated dishes were prepared as follows: agarose (Sigma), at a concentration of 2% in water, was heated for 15 min at 120 °C; while it was still hot, the same volume of 2 × HBSS was added, and 4 ml (for a 100 mm dish) or 0·5 ml (for a well of a 12-well plate) of the solution was added to each dish. The dish was left at room temperature until the agarose solidified. Dishes were washed with medium before use. To investigate development of follicles, cells cultured in agarose-coated dishes for 3 days were embedded in paraffin, sectioned (6 μm sections), and stained by periodic acid Schiff (PAS) procedure.

Preparation of thin sections for electron microscopy

The procedure for the fixation and embedding for electron microscopic examination was described previously (Cornford *et al.* 1993). At the end of culturing, the cell aggregates were collected by centrifugation and the pellet was fixed by the addition of 2% paraformaldehyde in 0·01 M sodium phosphate (pH 7·4) containing 0·05% glutaraldehyde, 0·9% NaCl, 0·15 mg/ml $CaCl_2$, and 0·2 M sucrose. After standing for 15 min on ice, the cell pellets were washed with phosphate-buffered saline, pH 7·0 (Gibco) and embedded in LR-white resin (Ted Tella Inc., Redding, CA, USA) (Cornford *et al.* 1993). Blocks of the plastic-embedded cells were sectioned and the ultra-thin sections were mounted on formvar-coated slot grids. Electron micrographs were taken with a JEOL electron microscope operating at 80 kV.

Tg production assay

Twenty-four-hour-conditioned medium was collected from individual plates and assayed for Tg secreted in the supernatants by a standard immunoradiometric assay method using a commercial kit (DPC, Los Angeles, CA, USA), according to the manufacturer's instructions.

Iodide uptake assay

To study thyroid follicles, cells in agarose-coated dishes were transferred to 1·5 ml microtubes, centrifuged and washed with HBSS. Then, the cells were incubated with 100 nmol/l $^{125}I^-$ (50 mCi/mmol) (ICN, Costa Mesa, CA, USA) in HBSS at 37 °C on agarose-coated 12-well plates. After 2 h incubation, the cells were collected in 1·5 ml microtubes, centrifuged, and rapidly rinsed with HBSS twice. The radioactivity of the pellet was measured with a γ-counter. For monolayer cells (including FRTL-5 cells), assay was performed as described previously (Saito *et al.* 1997). The radioactivity was normalized to the cellular protein content measured in the same cells using a Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA, USA). Non-specific binding of $^{125}I^-$ was determined in duplicate assays after 2 h in the presence of 30 μg/ml $KClO_4$ as previously described (Weiss *et al.* 1984), and this value was normalized to the cellular protein content.

Measurement of iodide organification and peroxidase activity

Follicle-forming cells were incubated with 100 nmol/l $^{125}I^-$ (50 mCi/mmol) (ICN) in HBSS at 37 °C for 2 h. After the incubation, the cells were rinsed with 1 ml HBSS, and the radioactivity of the cells was counted by γ-counter. Then, the cells were suspended in 1 ml HBSS, sonicated and precipitated by adding 1 ml 20% trichloroacetic acid (TCA), followed by centrifugation at 700 ***g*** for 10 min. After washing the precipitate with 10% TCA solution, the radioactivity of TCA-precipitated protein was counted and the value was normalized to the cellular protein content measured in the same cells with the Bio-Rad protein assay.

Northern blot analysis

Total RNA (5 μg) prepared with Trizol reagent (Gibco) from cultured cells was separated on a 1% agarose gel containing formaldehyde and transferred to a nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH, USA) as described previously (Sambrook *et al.* 1989). A human NIS cRNA probe was prepared by the *in vitro* transcription of pcDNA3 containing the human NIS cDNA, provided by Drs T Endo and T Onaya (Yamanashi Medical University, Japan), with SP6 RNA polymerase (Promega) as described previously (Saito *et al.* 1997). The filters were hybridized with the probe. Intensities of 28S ribosomal RNA in the agarose gels and NIS mRNA signals on the blots were quantitated on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

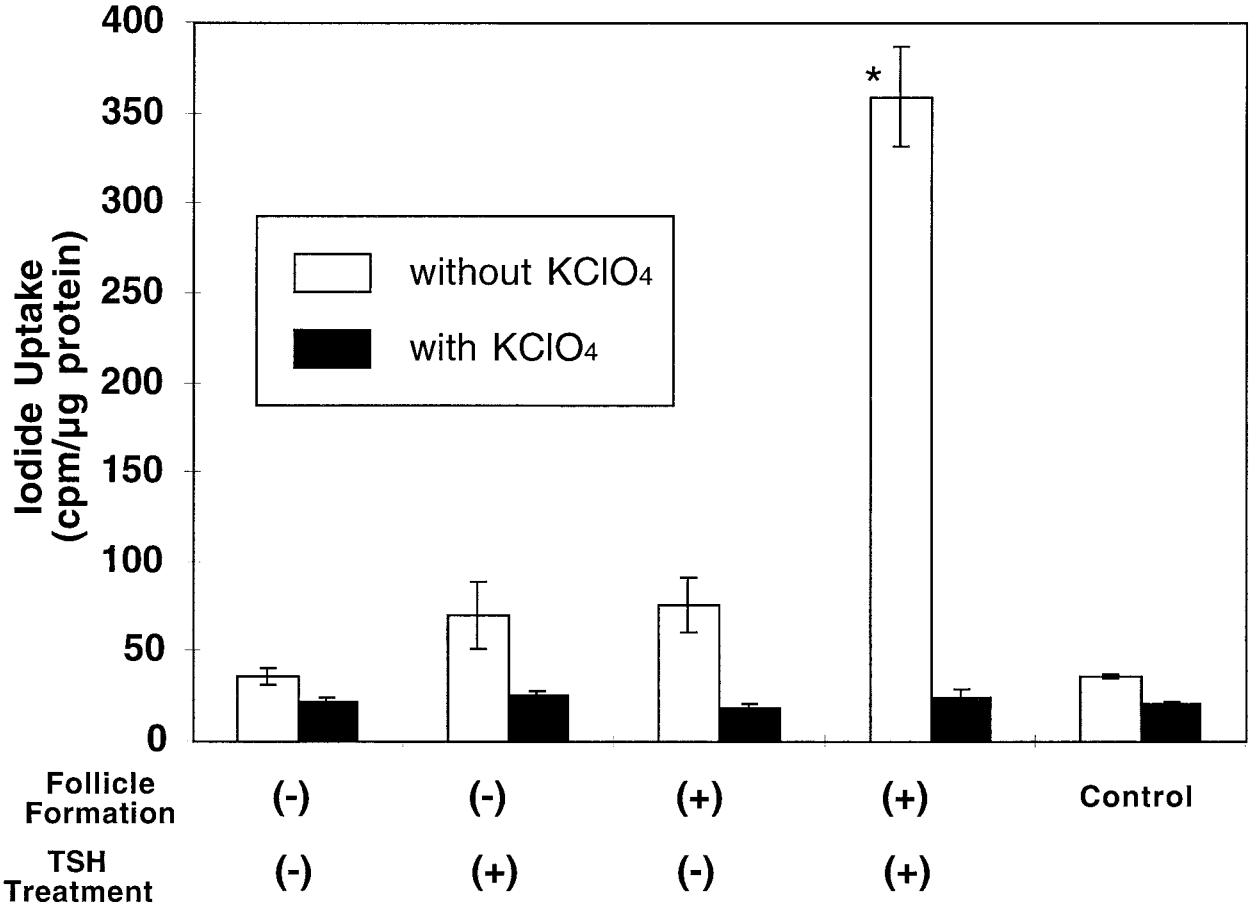


Figure 2 Iodide uptake activity in normal human primary thyroid cells in the monolayer and follicle-forming conditions. Control: monolayer cells were maintained in the basic culture medium for 34–56 days and then the iodide uptake assay was performed. Follicle formation (−) (monolayers): after long-term culture (34–56 days) in the basic culture medium, the medium was switched to the follicle-induction medium (serum-free) and cells were incubated with or without 0·1 mU/ml bovine TSH in the monolayer condition for 3 days. Then the assay was performed. Monolayer cells can survive in the follicle-induction medium (free of hypothalamus and pituitary extracts) for only 3–4 days. Follicle formation (+): after long-term culture (34–56 days) in the basic medium, cells were induced to form follicles. They were incubated with or without 0·1 mU/ml bovine TSH for 3–5 days and subjected to iodide uptake assay. Non-specific binding of $^{125}I^-$ was determined in duplicate assays after 2 h in the presence of 30 µg/ml $KClO_4$ (■). Values are the mean ± S.E. ($n=5$). *Significantly greater ($P<0·001$) than other conditions.

Western blot analysis

Post-nuclear membrane fractions were prepared from monolayer cells or follicle forming cells as described previously (Kogai *et al.* 1997). The lysates (20 µg/lane) were subjected to electrophoresis on a 10% polyacrylamide gel containing 0·1% SDS, and transferred to a nitrocellulose filter. Immunoblot analysis was performed with antiserum (1:500) generated with glutathione-S-transferase/human NIS (amino acids 466–522) fusion protein, provided by Drs T Endo and T Onaya (Yamanashi Medical University, Japan) (Saito *et al.* 1998) and ^{125}I-conjugated anti-rabbit antibody (ICN). The abundance of immunoreactive protein was quantitated with NIH image version 1·6 (NIH).

Table 2 Effect of FCS on iodide uptake activity in follicle-forming cells. Cells were cultured in the basic medium for 32 days and then seeded in agarose-coated dishes. After 5 days treatment with or without 1% FCS in the follicle-induction medium (containing 0 or 0·1 mU/ml TSH), the iodide uptake assay was performed as described in Materials and Methods. Values are the means ± S.E. ($n=3$)

	TSH (mU/ml)	
	0	0·1
FCS-free (c.p.m./µg protein	57·8 ± 22·1	334 ± 54·7
1% FCS (c.p.m./µg protein)	33·8 ± 0·44	276 ± 57·4

Statistical analysis

Results presented are representative of at least three replicate experiments. Error bars represent 1 S.E. When

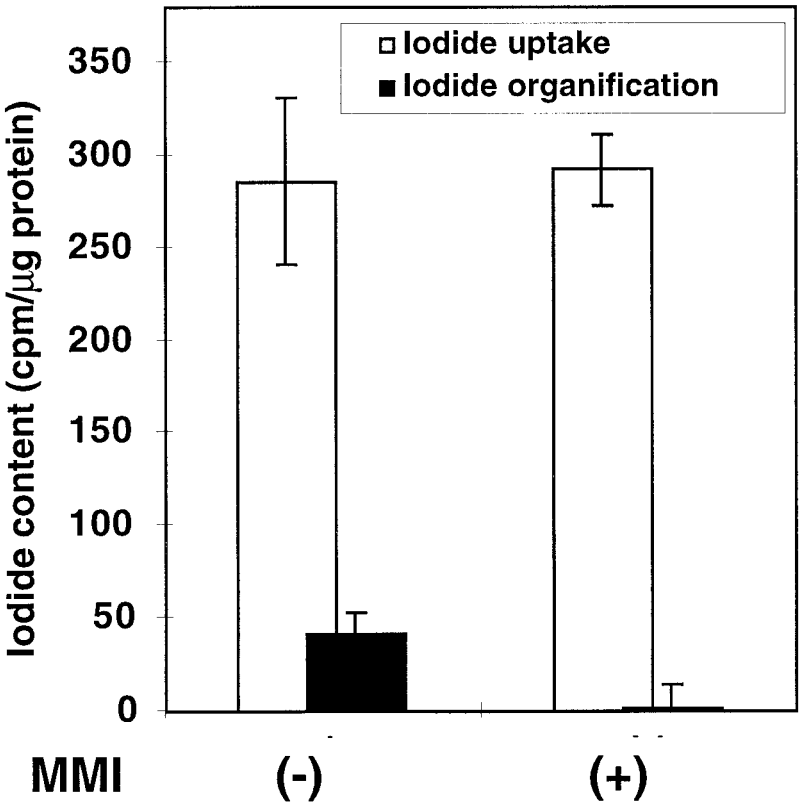


Figure 3 Iodide organification in TSH-stimulated follicle-forming cells. Cells were maintained as monolayers for 36 days and induced to form follicles with 0·1 mU/ml bovine TSH as described in the legend for Fig. 2. Cells were incubated for 2 h in HBSS containing $Na^{125}I$ with 0 or 1 mM MMI as indicated above. After the incubation, iodide accumulated into the cell aggregates was counted (shown as iodide uptake), and TCA precipitation of the cell lysate was carried out. Precipitated iodide was counted (shown as iodide organification). Non-specific binding of $^{125}I^-$ was determined in duplicate assays after 2 h in the presence of 30 μg/ml $KClO_4$, and this value was normalized to the cellular protein content and subtracted from the values measured as described previously (Weiss *et al.* 1984).The iodide content was normalized for protein content of the cells. Values are the mean ± S.E. ($n=3$).

error bars are not visible, they are obscured by the symbol for the mean. Results were analyzed by Student's *t*-test. When multiple comparisons were made, statistical significance was determined by ANOVA with the Bonferroni/Dunn *post-hoc* test. $P<0·05$ was considered statistically significant.

Results

Follicle reconstitution by long-term cultured normal human thyroid cells

Human normal thyroid cells can be maintained as monolayer cells in the basic culture medium (containing 5% FCS and bovine hypothalamus and pituitary extracts) for about 3–4 months as previously described (Curcio *et al.* 1994) (Fig. 1A). Variable levels of thyroglobulin (Tg) were secreted into the media after 1–2 months of culture (Table 1). These data are consistent with the findings of a previous report (Curcio *et al.* 1994) and confirmed that the primary thyroid cells in culture we studied were functional. When cells were seeded in agarose-coated dishes and maintained in the follicle-induction medium (free of hypothalamus and pituitary extracts), they associated into globular aggregates in 18–24 h (Fig. 1B). PAS staining demonstrated numerous follicles with PAS-positive lumens within the aggregates (Fig. 1C), suggesting the presence of colloid. The electron micrographs showed that the electron-dense microvilli were oriented towards the inside of the lumen, but not at the outer surface (Fig. 1D, E). These data suggest that the induction of follicles from monolayer cells is structurally similar to that of the

functional thyroid follicle, as indicated by cell polarity and central colloid accumulation. The absence or presence of TSH and FCS (up to 1%) in the medium did not affect the follicle formation significantly under light microscopic examination (data not shown). The follicles could be maintained for at least 8 days with or without TSH. In contrast, FRTL-5 cells treated under the same conditions were not observed to make follicles. This observation is consistent with those of a recent study attempting to induce follicles in FRTL-5 cells using a different approach (Burgi-Saville *et al.* 1998).

Iodide uptake by monolayer cells and follicle-forming cells

The iodide uptake activity of long-term cultured primary human thyroid cells was relatively low (Fig. 2, control), and the addition of TSH (10 μU/ml, 0·1 mU/ml, or 1 mU/ml) to the basic medium did not further increase the iodide uptake (data not shown). When cells were maintained in follicle-induction medium (serum-free) for 3 days in the monolayer condition, TSH increased iodide uptake activity about 2·3-fold, although this was not statistically significant (Fig. 2). Follicle induction alone (without TSH-treatment) also increased iodide uptake activity 2·7-fold, although the difference was not significant. In contrast, when the cells formed follicles under serum-free condition, TSH markedly increased iodide uptake about 4·4-fold (Fig. 2). The combination of follicle induction and TSH treatment significantly stimulated iodide uptake activity (12·0-fold) compared with that in untreated monolayers (Fig. 2). Two-factor factorial ANOVA indicated that the addition of TSH and follicle induction synergistically stimulated iodide uptake in human primary thyroid cells ($P<0·001$). The tested cells were maintained in the basic medium for 34–56 days. However, different periods of culture did not change the iodide uptake activity. The iodide uptake levels in follicles with or without TSH were similar to those of FRTL-5 cells with and without TSH (data not shown). Although the use of media with 1% FCS slightly decreased the iodide uptake by follicles, the difference was not significant (Table 2).

Iodide organification does not affect the uptake of iodide by follicle-forming cells

To evaluate whether iodide organification affects the uptake of iodide in follicle-forming cells, TSH-treated follicle-forming cells were incubated with $Na^{125}I$ and 0 or 1 mM mercaptomethylimidazol (MMI), and TCA precipitation of the cell lysate was performed. As shown in Fig. 3, 17% of iodide in follicle-forming cells was organified without MMI. Although MMI completely blocked iodide organification in follicle-forming cells, iodide uptake was not changed by MMI treatment (Fig. 3).

NIS mRNA and protein levels in monolayer cells and follicle-forming cells

As shown in Fig. 2, follicle induction with TSH stimulation markedly stimulates iodide uptake activity in long-term cultured normal human thyroid cells. To determine the mechanism involved in the up-regulation of iodide uptake, we determined NIS mRNA levels by Northern blot analysis of total RNA utilizing a human NIS cRNA probe. When the cells were stimulated by 0·1 mU/ml TSH, the NIS mRNA levels in the monolayer cells and follicle-forming cells were increased 3·4- to 5·5-fold (Fig. 4). TSH significantly stimulated NIS gene expression in both monolayer and follicles, to a similar level. We next determined the levels of NIS protein. Western blot analysis of post-nuclear fractions with antiserum to human NIS detected an approximately 77 kDa immunoreactive protein in both monolayer cells and follicle-forming cells. As described previously (Levy *et al.* 1997, 1998, Paire *et al.* 1997, Saito *et al.* 1998), the 77 kDa-stained band indicates glycosylated NIS (Fig. 5A). NIS protein was markedly stimulated by TSH treatment in monolayer and follicles (∼4·9- and ∼6·8-fold respectively). The levels of NIS protein were very similar in TSH-stimulated monolayers and follicles, but the functional uptake of iodide was much greater in follicles, as shown in Fig. 2.

Discussion

It has been reported that follicle structure or cell polarity is required for differentiated thyroid function, especially transcellular iodide transport in primary cultures of human (Dickson *et al.* 1981, Roger *et al.* 1988, Kraiem *et al.* 1991, Eggo *et al.* 1996), porcine (Nakamura *et al.* 1990, Takasu *et al.* 1992, Nasu & Sugawara 1994), and ovine (Becks *et al.* 1992) thyroid cells. Conventional methods for investigating iodide transport in human primary culture cells have used thyrocytes cultured for no longer than 10 days (Dickson *et al.* 1981, Roger *et al.* 1988, Kraiem *et al.* 1991, Eggo *et al.* 1996, Saito *et al.* 1997, 1998). In the present study, we showed that long-term cultured normal human thyroid cells can display iodide uptake activity up to 2 months after the initiation of cell culture, when they are seeded on agarose-coated dishes.

TSH/cAMP has previously been shown to stimulate iodide uptake and NIS mRNA expression in FRTL-5 cells (Kogai *et al.* 1997, Paire *et al.* 1997, Ajjan *et al.* 1998, Pekary *et al.* 1998) and primary cultures of human thyroid cells maintained for 7–10 days (Saito *et al.* 1997). The up-regulation of iodide uptake and NIS mRNA and protein expression by TSH in our follicle-forming cells is consistent with these previous observations.

It was reported that the presence of 1% serum reduced the iodide uptake stimulated by TSH (Roger *et al.* 1988, Kraiem *et al.* 1991, Saito *et al.* 1997). In the culture method used by Roger *et al.* (1988), follicles released from

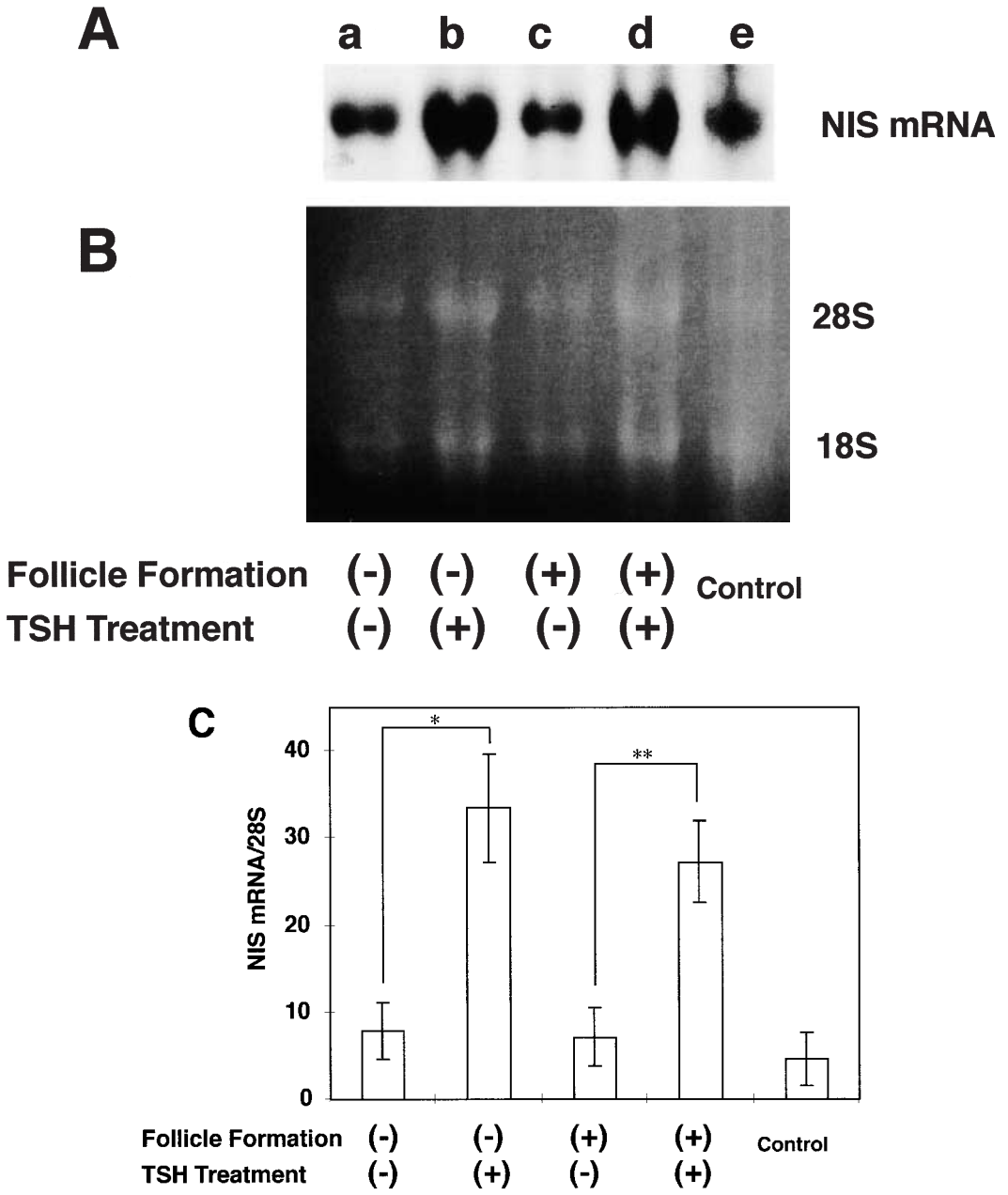


Figure 4 Expression of NIS mRNA in long-term cultured normal thyroid cells. Human primary culture cells were maintained in the basic medium for 32–56 days and then seeded in normal dishes (follicle formation (−); lanes a, b) or agarose-coated dishes (follicle formation (+); lanes c, d) in which they reconstituted follicles. After incubation for 3 days (follicle formation (−)) or 3–5 days (follicle formation (+)) in the follicle-induction medium (serum-free) with (lanes b, d) or without (lanes a, c) TSH, total RNA was extracted and Northern blot analysis (5 µg total RNA, each lane) was performed. In lane e, total RNA from cells maintained in the basic medium was directly applied. (A) Hybridization with a [^{32}P]-labeled human NIS RNA probe. (B) Ethidium bromide-stained agarose gel. (C) Radioactivity of the blots was quantified and normalized to the fluorescence intensity of the corresponding 28S ribosomal RNA band. Control, NIS mRNA level in the basic medium-treated cells. Values are mean ± S.E. (n=3). *P<0·02, **P<0·04.

thyroid tissue by collagenase and some other enzymes are seeded with 1% FCS, and the medium is changed 12 h after seeding, when the follicles attach to the dish (non-coated) but the follicle structure is not yet destroyed (Roger *et al.* 1988). Long exposure (over 12 h) of released follicles to FCS made cells grow as monolayers and

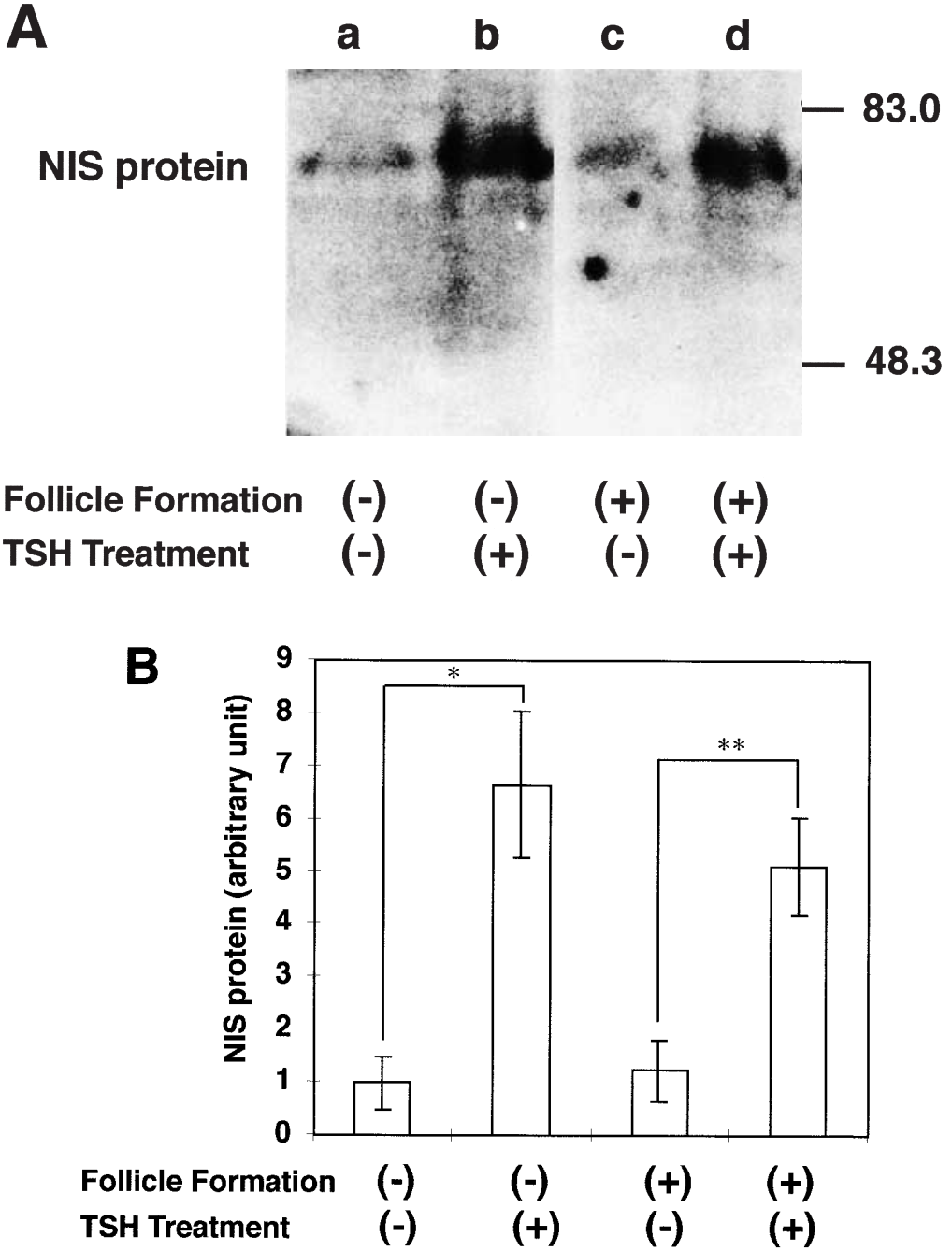


Figure 5 Western blot analysis of NIS protein in long-term cultured normal human thyroid cells. (A) Post-nuclear membrane fractions prepared from monolayer cells (follicle formation (−); lanes a, b) and follicle-forming cells (lanes c, d) were subjected to Western blot analysis with antiserum (1:500) to human NIS. Cells were prepared as described in the legend to Fig. 3. (B) Quantitative analysis of the data in A. Data are the mean ± S.E. ($n=3$) and are expressed relative to the mean of the data in follicle formation (−) and TSH treatment (−). *$P<0{\cdot}02$, **$P<0{\cdot}04$.

irreversibly abolished the iodide uptake activity (Roger *et al.* 1988). In the present study, we found no significant difference between the cells cultured with 1% FCS and those without FCS when they formed follicles, whereas iodide uptake activity was markedly reduced in the monolayer condition. Therefore, we conclude that, if

the cells form follicle structures, iodide can be trapped in the cells, even if they are exposed to low concentrations of serum in the media.

Our Northern blot analysis indicated that NIS mRNA levels in the monolayer cells and the follicle-forming cells were almost equal. Furthermore, Western blot analysis indicated that induction of follicles did not change NIS protein concentrations, and that NIS protein in both types of cells was glycosylated just as in primary cells cultured for only 10 days (Saito *et al.* 1997, 1998). TSH markedly stimulated NIS mRNA and protein in both monolayer and follicles. Significant stimulation of NIS function, however, was only seen with follicles. Therefore, the defect of iodide uptake in the monolayer cells may result from failure of post-transcriptional events, such as failure of translocation of the NIS protein or NIS regulatory protein. The statistical analysis indicated that the effect of follicle induction on iodide uptake was synergistic with that of TSH. This may suggest the existence of a TSH-dependent NIS regulatory factor, which has been proposed previously (Kaminsky *et al.* 1994, Kogai *et al.* 1997, Saito *et al.* 1998). In the monolayer condition, this factor might not be effective. Another possibility is that the stimulation of iodide uptake by follicle formation and TSH may be the result of activation of Na–K-ATPase. TSH can stimulate Na–K-ATPase in FRTL-5 rat thyroid cells (Pressley *et al.* 1995) and porcine primary thyroid cells (Paire *et al.* 1998). The location of the enzyme in follicles of porcine primary thyroid cells is on the basolateral membrane (Kuliawat *et al.* 1995). It is possible that follicle formation, TSH stimulation, or both, increased Na–K-ATPase activity in our culture system, which may contribute to the stimulation of iodide transport into cells.

Iodide accumulation in thyroid follicular cells is not only a function of activity of the NIS, but may also be influenced by the extent of iodide organification. It has been reported that the formation of protein-bound iodine (PBI) is rapid (within 10 min) after iodide uptake, and that PBI represents 50–70% of total concentrated iodide in primary human follicle culture from colloid goiter (Kraiem *et al.* 1991). In contrast, primary thyroid cells grown in monolayer exhibit very low PBI formation (Dickson *et al.* 1981). PBI in follicle-forming cells in our culture system was $\sim$17% of total concentrated iodide. Iodide uptake, however, was not decreased by MMI at a concentration that completely blocked iodide organification.

The accumulation of iodide by follicles shown in the present study may reflect iodide accumulation both in follicular cells and in the follicular lumen. In the porcine thyroid follicle culture system, iodide efflux on the apical membrane has been shown to be stimulated by TSH/cAMP (Nilsson *et al.* 1990, 1992). In our culture system, TSH may also stimulate iodide efflux into the lumen. Polarized functions, such as iodide efflux into follicular lumina, may be restored in our follicle culture and contribute to the increased iodide concentration.

In summary, we investigated the mechanism of iodide transport in long-term cultured human thyroid primary cells. TSH-stimulated increase in NIS protein is necessary, but not sufficient, to stimulate iodide uptake in primary thyroid cells. Follicle formation has significant effects on the function of NIS protein. This system should facilitate the study of the precise mechanism of iodide transport and may produce a practical approach to evaluate iodide transport in thyroid diseases.

Acknowledgements

We are grateful to Drs Toyoshi Endo and Toshimasa Onaya (Yamanashi Medical University, Yamanashi, Japan) for providing human NIS cDNA and anti-serum to human NIS. We also thank Birgitta Sjostrand, Cathleen Enriquez (Brain Research Institute, UCLA School of Medicine, CA, USA) and Nataliya Bufius (VivoRx, Santa Monica, CA, USA) for technical assistance and Drs Katsuaki Motomura and A Eugene Pekary (West Los Angeles VA Medical Center, Los Angeles, CA, USA) for useful discussions. This work was supported by grants from Knoll Pharmaceutical Company, Thomas B Rosenberg, VA Medical Research Funds (J M H and G A B), NIH NS 37360 (E M C and S H), and NIH DK 43714 (G A B).

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Received 22 July 1999
Revised manuscript received 2 March 2000
Accepted 23 May 2000