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Thyrotropin Prevents Apoptosis by Promoting Cell Adhesion and Cell Cycle Progression in FRTL-5 Cells

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ABSTRACT

Apoptosis has been shown to be involved in endocrine tissue homeostasis as well as regression due to hormone deprivation. The goal of this study was to induce apoptosis and to investigate a potential role of TSH as a survival factor in thyroid follicular cells (FRTL-5) *in vitro*. Our results indicated that FRTL-5 cells underwent anchorage-dependent apoptosis when plated in the absence of serum and hormones, but when the cells became attached to the substrate by addition of TSH in the medium, apoptosis was prevented. The apoptosis was evaluated by positive terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling staining, typical apoptotic bodies by electron microscopy, DNA ladder by gel electrophoresis, and subdiploidy by propidium iodide-stained flow cytometry. TSH was shown to prevent apoptosis and maintain cell viability. cAMP partly mimicked this effect, which was inhibited by a specific inhibitor of protein kinase A, H-89. While investigating the mechanisms of apoptosis, we observed that the phosphorylated focal adhesion kinase was strengthened by TSH. Furthermore, FRTL-5 cells were found to undergo growth arrest in the G_1 phase in the absence of TSH, accompanied by an elevated level of cyclin-dependent kinase inhibitor, p27, and a decreased level of cyclin D. In contrast, TSH promoted transition from G_1 to S phase by decreasing P27 protein and increasing cyclin D expression. We concluded that in addition to regulating growth and differentiation, TSH may function as a survival factor in thyroid cells by preventing anchorage-dependent apoptosis in FRTL-5 cells partly via the cAMP pathway. (*Endocrinology* **140:** 5962–5970, 1999)

MOST MAMMALIAN cells are programmed to depend on a specific set of signals for survival. When deprived of an appropriate signal, a suicide program of cells might be activated that causes cells to kill themselves by a process called programmed cell death. Different types of cells are thought to require different sets of survival signals. Programmed cell death, a kind of apoptosis, has been shown to be involved in the homeostasis of endocrine tissue function as well as in the regression due to hormone deprivation. These phenomena have been observed in various endocrine organs, *e.g.* rat prostate underwent apoptosis after orchidectomy (1), apoptosis was induced in androgen-dependent mouse mammary cell line by the deprivation of testosterone (2), and apoptotic cell death was associated with the initiation of ovarian follicular atresia (3). In the thyroid gland, apoptosis may be involved in the destructive mechanisms of autoimmune thyroiditis and cancer cell death in thyroid carcinoma. In contrast, no apoptosis was detected in Grave's disease, which is characterized by hyperthyroidism induced by the stimulation of TSH receptor (4). Apoptosis was induced in primary cultured dog thyroid cells by deprivation of TSH (5), which plays a central role in the regulation of thyroid follicular cell structure and function. In addition, TSH was reported to protect thyroid cells from apoptosis by down-regulating the expression of Fas antigen (6).

To further determine a potential role of TSH in regulating the apoptosis of thyroid follicular cells, we attempted to induce apoptosis by the deprivation of serum and hormones in a rat thyroid cell line, FRTL-5 cells. FRTL is a functional thyroid follicular cell line that was established from normal Fisher rat thyroid and requires six hormones, including TSH, for growth (7). Therefore, this cell line is thought to be an ideal model for the investigation of hormone-dependent apoptosis of the thyroid follicular epithelial cells. FRTL-5 cells that were cloned from FRTL cells share many features with FRTL cells, such as the expression of TSH receptor, TSH-dependent growth, synthesis and secretion of thyroglobulin, active trapping of iodine, *etc.* TSH was particularly important for the growth of FRTL/FRTL-5 cells. Its withdrawal was thought to be an effective means of cell synchronization (8). FRTL cells were found to undergo dramatic morphological changes and ceased growth when cultured in the absence of TSH (9). However, the occurrence of apoptosis in FRTL-5 cells induced by deprivation of TSH has not been reported to date. Recently, there was a report that apoptosis was induced in quiescent FRTL-5 cells by transforming growth factor-β1. Transforming growth factor-β1 was found to prevent resting cells from entering the cell cycle when stimulated by serum and a mixture of five hormones, which was added in the usual culture of FRTL-5 cells, but not by TSH and insulin-like growth factor I (IGF-I), accompanied by a reduction in cyclin D and an increase in c-*myc* (10).

In this study, we demonstrate for the first time that FRTL-5 cells underwent anchorage-dependent apoptosis in the absence of serum and hormones as evaluated by several apoptosis features, and that TSH prevented apoptosis by promoting cell-matrix adhesion and cell cycle progression partly via the cAMP pathway.

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Materials and Methods

Cell culture

FRTL-5 cells were cultured by the usual method in Ham's F-12 medium containing 5% calf serum (Life Technologies, Inc., Grand Island,

NY) and a mixture of six hormones [six hormones in 5% calf serum (6H5)] including 10 μg/ml bovine insulin, 10 nM hydrocortisone, 5 μg/ml transferrin, 10 ng/ml somatostatin, 10 ng/ml glycyl-L-histidyl-L-lysine acetate and 10 mU/ml bovine TSH. All of the hormones were obtained from Sigma Chemical Co. (St. Louis, MO). FRTL-5 cells were grown in 75-ml flasks (Falcon) and passaged by trypsinization every week.

To induce apoptosis, we examined two culture conditions: 1) FRTL-5 cells were cultured in 6H5 for 3 days to reach subconfluence and washed with PBS (−), then further cultured in Ham's serum-free medium containing 0.2% BSA (Wako, Japan), which was referred to as 0H, for up to 1 week; 2), FRTL-5 cells were cultured in 6H5 medium for 3 days to reach subconfluence and washed with PBS (−), then starved in 0H for 1–2 days. The cells were detached by trypsin treatment and plated at 5×10^5/ml in 0H with or without 10 mU/ml TSH or 1 mM $(Bu)_2$cAMP (Wako) for the indicated times. For blockage of cell-matrix adhesion, FRTL-5 cells were cultured in 2% agarose-coated dishes or flasks.

Cell viability evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT) assay

The MTT assay was carried out according to the manufacturer's instructions (Chemicon International, Inc., Temcula, CA). Briefly, FRTL-5 cells were passaged into 96-well tissue plates (Falcon) at about 0.5×10^5/100 μl and cultured in 0H with or without 10 mU/ml TSH or 1 mM cAMP for up to 72 h. To determine the contribution of the cAMP-dependent pathway to the antiapoptotic effect, FRTL-5 cells were pretreated with a specific protein kinase A inhibitor H-89 (*N*-[2-(*p*-bromocinnamylamino)ethyl-5-isoquinolinesulfonamide; BIOMOL, Plymouth Meeting, PA; 25 μM) for 30 min (11). On the day of the experiment, 10 μl 5 mg/ml MTT were added to each pore and cultured for an additional 4 h. Then 100 μl isopropanol with 0.04 N HCl was added and pipetted thoroughly. Within 1 h, the value of absorbance expressed by the OD at 570 nm by spectrophotometry was measured on a plate reader (SPECTRAmax 340, Molecular Devices, Menlo Park, CA). Cell viability at the indicated times was expressed as a percentage of the absorbance of cells at time zero. Data were presented as mean ± SD of triplicate determinations from three independent experiments.

Specific labeling of nuclear DNA fragmentation by the terminal deoxynucleotidyl transferase-mediated deoxy-UTP nick end labeling (TUNEL) method (12)

FRTL-5 cells were collected by cell scrapers and washed with PBS (−). The cells were then fixed with 4% paraformaldehyde for 15 min at room temperature and spotted onto silane-coated slides. After washing with PBS, the cells were treated with 2 μg/ml proteinase K for 15 min at room temperature. After washing with PBS and rinsing with TDT buffer (30 mM Trizma base, 140 nM sodium cacodylate, and 1 mM cobalt chloride), pH 7.2, the cells were incubated with 0.3 U/μl TDT and 0.01 mM biotin-16-deoxy-UTP (Sigma Chemical Co.) in the above buffer for 1 h at 37 C. The reaction was blocked by TB buffer (300 mM sodium chloride and 30 mM sodium citrate) for 15 min at room temperature. After washing with PBS, the cells were incubated with fluorescein-labeled streptavidin in 1% BSA/PBS containing 2 μg/ml 4′,6′-diamido-2 phenylindole hydrochloride (DAPI; Boehringer Mannheim Biochemica) for 60 min at room temperature. After washing with PBS, the slides were sealed with 50% glycerol in PBS. Photographs were taken under a fluorescence microscope (Nikon, Tokyo, Japan).

Transmission electron microscopy

FRTL-5 cells were collected by cell scrapers and centrifuged at 1500 rpm for 10 min. The pellets were fixed with 2.5% glutaraldehyde in PB buffer, then postfixed with 1% OsO_4 in PB buffer. After having been dehydrated by an ethanol series, the cells were embedded in Epon 812. Ultrathin sections were cut by an ultramicrotome (LKB, Rockville, MD), stained with uranyl acetate and lead citrate, and observed under an electron microscope (JEM-1005X, JEOL, Tokyo, Japan) at 80 kV.

DNA fragmentation analysis by agarose gel electrophoresis

Low mol wt DNA was isolated as described previously (13). Cells (2×10^6) were collected by cell scrapers and washed with PBS (−). The resulting cell pellets were resuspended in lysis buffer [10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5% Triton X-100] and centrifuged at about $13{,}000 \times g$ for 20 min at 4 C to separate the fragmented DNA (soluble) from intact chromatin. The supernatant was treated with 0.1 mg/ml deoxyribonuclease-free ribonuclease for 1 h at 37 C, followed by 0.2 mg/ml proteinase K and 1% SDS for 2 h at 50 C. The DNA was then extracted with phenol and phenol/chloroform and precipitated overnight with isopropanol at −20 C. The DNA samples were analyzed with 1.8% agarose gel.

Flow cytometric analysis for DNA fragmentation and cell cycle

Flow cytometric analysis was performed as described previously (14). Briefly, FRTL-5 cells were cultured in tissue flasks under the conditions described above. The cells were collected by cell scrapers and centrifuged. Then the cell pellets were gently resuspended in 1.2 ml hypotonic fluorochrome solution containing propidium iodide (PI; 50 μg/ml) in 0.1% sodium citrate and 0.1% Triton X-100 and stained overnight at 4 C. The cells were analyzed on a fluorescence-activated cell sorter (Becton Dickinson and Co., Rutherford, NJ). The data were analyzed using ModFitLT V1.00 (Macintosh) software.

Indirect immunofluorescence

Antibodies were obtained from the following sources: rabbit antimouse laminin antibody (Sigma Chemical Co.), rabbit antimouse type IV collagen (LSL, Japan), rabbit antirat fibronectin (Life Technologies, Inc.), rabbit antihuman α_5 integrin (Chemicon), mouse antihuman p27 monoclonal antibody (Transduction Laboratories, Inc., Lexington, KY), anti-cyclin D1 and 2/bcl-1 monoclonal antibody (MBL, Japan), antifocal adhesion kinase [anti-FAK; monoclonal antibody from Upstate Biotechnology, Inc. (Lake Placid, NY), and polyclonal antibody from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)], and antiphosphorylated tyrosine (4G10, Upstate Biotechnology, Inc.; PY20, Transduction Laboratories, Inc.).

The cells for immunofluorescence tests were seeded onto glass coverslips of 1.2 cm in diameter, fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, and permeabilized with 0.1% Triton X-100 in PBS for 30 min. For α_5 integrin, the cells were fixed with cold 100% acetone for 3 min. The cells were incubated with primary antibody in 1% BSA/PBS overnight at 4 C. The coverslips were then washed with PBS and allowed to react with fluorescein-labeled secondary antibody (DAKO Corp.) for 45 min at room temperature. After washing with PBS, the coverslips were mounted on slides using 50% glycerol in PBS and observed using a confocal scanning microscope (Leica Corp., Heidelberg, Germany). For actin filament staining, paraformaldehyde-fixed and permeabilized cells were stained with 2 U/ml rhodamine-phalloidin (Molecular Probes, Inc., Eugene, OR) for 1 h at room temperature.

The cells stained by anti-α_5 integrin antibody were also analyzed using a fluorescence-activated cell sorter (Becton Dickinson and Co.).

Immunoprecipitation and Western blot analysis

FRTL-5 cells were seeded in 75-ml flasks in 6H5 medium for 3 days to subconfluence and starved in 0H for 1–2 days. The cells were then detached by trypsin treatment and seeded at about 5×10^6/10 ml in 10-cm dishes in 0H with or without 1 mU/ml TSH or 1 mM cAMP for the indicated times. The cells were collected by cell scrapers and washed once with cold PBS. After centrifuging, the cell pellet was lysed at 4 C in 0.3 ml lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM sodium vanadate, 1 mM EDTA, 1% Triton X-100, 25 μg/ml leupeptin, and 0.5 mM phenylmethylsulfonylfluoride; sonicated briefly; and centrifuged. After adding 0.1 ml of 3 × Laemmli's sample buffer to 0.2 ml of the above suspension, the mixture was incubated for 3 min at 100 C. Protein assay was carried out by the method of Lowry. For immunoprecipitation, the lysate (~500 μg protein) was precleared with 50 μl protein A-Sepharose beads, and the supernatant was incubated with 3 μg anti-FAK monoclonal antibody for 2 h at 4 C. The immunocomplex was recovered by the addition of 50 μl protein A-Sepharose beads, incubated for 1 h at 4 C, and centrifuged. The beads were washed with lysis buffer three times and eluted by boiling in sample buffer.

For Western blot analysis, approximately 50 μg immunoprecipitated

protein or whole cell lysate were run on 8% or 12% SDS-PAGE and transferred to a polyvinylidene difluorene membrane (ATTO, Japan). Blots were blocked with 1% BSA in PBS overnight at 4 C. After washing with PBS containing 0.4% Tween-80 three times, the blots were incubated with 1 μg/ml antiphosphotyrosine antibody (4G10 and PY20) or anti-FAK polyclonal antibody for 2 h, rinsed, and incubated with a 1:10,000 dilution of horseradish peroxidase-labeled goat secondary antibody for 2 h. The blots were again rinsed and visualized using enhanced chemiluminescence (ECL kit, Amersham Pharmacia Biotech, Arlington Heights, IL) according to the manufacturer's instructions. For cell cycle-relevant proteins, the membrane was first immunoblotted with a monoclonal antibody against cyclin D (MBL) at a dilution of 1:100. After having been developed by ECL, the membrane was deprobed using stripping buffer (62.5 mM Tris-HCl, 2% SDS, and 0.4 M 2-mercaptoethanol) at 60 C for 30 min and reprobed by a monoclonal antibody against p27 (Transduction Laboratories, Inc.) at a dilution of 1:2500.

Statistics

Statistical analysis was performed using ANOVA, followed by Fisher's protected least significant difference test (PLSD; StatView 4.0). $P < 0.01$ was selected as the level of significance.

Results

Induction of apoptosis by deprivation of serum and TSH from culture medium

As indicated in *Materials and Methods*, we examined two culture conditions to induce apoptosis. Although some apoptotic cells were detected by TUNEL staining (data not shown) and electron microscopy (Fig. 1) when cultured by method 1, in which FRTL-5 cells had reached subconfluence in the presence of serum and hormones, then starved in 0H for up to 1 week, the percentage of apoptotic cells was quite low as evaluated by flow cytometry (not more than 20%). It was difficult to induce apoptosis by deprivation of serum and hormones if FRTL-5 cells became attached to the substrate, suggesting that cell-matrix adhesion might play an important role in maintaining the viability of FRTL-5 cells. Therefore, we tried to induce apoptosis by method 2, *i.e.* deprivation of serum and hormones as soon as subpassage was performed (see *Materials and Methods*). Sustained cell attachment was not obtained in the absence of TSH. Most cells floated in the serum-free medium and seemed to undergo apoptosis after 24 h (Fig. 2a). In contrast, in the presence of TSH, the cells attached to the polystyrene substrate and survived, forming follicle-like colonies, although the cells did not spread well without serum (Fig. 2b). cAMP showed an effect similar to TSH on maintenance of cell adhesions and cell viability (data not shown). Because apoptosis seemed to be satisfactorily induced by the latter method, we used this culture condition throughout the following experiments.

We next tried to evaluate the cell viability quantitatively by MTT assay. MTT is a yellow substance that is cleaved by living cells to yield a dark blue formazan product. The MTT dye reduction assay measures mitochondrial function and can detect the onset of cell death earlier than dye exclusion methods. The cell viability and cell number are proportional to the value of absorbance measured by spectrophotometry at 570 nm. TSH has been demonstrated to be an important mitogen for FRTL-5 cells (15, 16). It increased DNA synthesis and cell number mainly via the cAMP pathway. However, we did not observe a significant increase in cell number during the first 48 h culture after starved FRTL-5 cells were plated in 0H in the presence of TSH or cAMP (data not shown). This was consistent with the previous report, in which there was a 48-h lag period for DNA synthesis after readdition of TSH to starved FRTL-5 cells (16). As a result, MTT assay in our study mainly evaluated the viability of FRTL-5 cells during the first 48 h culture after subpassage.

The results showed that the cell viability was significantly reduced time dependently when FRTL-5 cells were cultured in 0H without TSH. In contrast, TSH and cAMP apparently maintained the viability of FRTL-5 cells during the first 48 h of culture ($p < 0.0001$; Fig. 3a). The apparent increase in cell viability at 72 h might result from the increase in cell number due to the mitogenic effect of TSH or cAMP. To further determine the contribution of the cAMP/PKA pathway to the maintenance of cell viability, we pretreated FRTL-5 cells with a specific protein kinase A inhibitor, H-89, 30 min before TSH or cAMP challenge. H-89 (25 μM) almost completely inhibited the effect of TSH or cAMP on the maintenance of

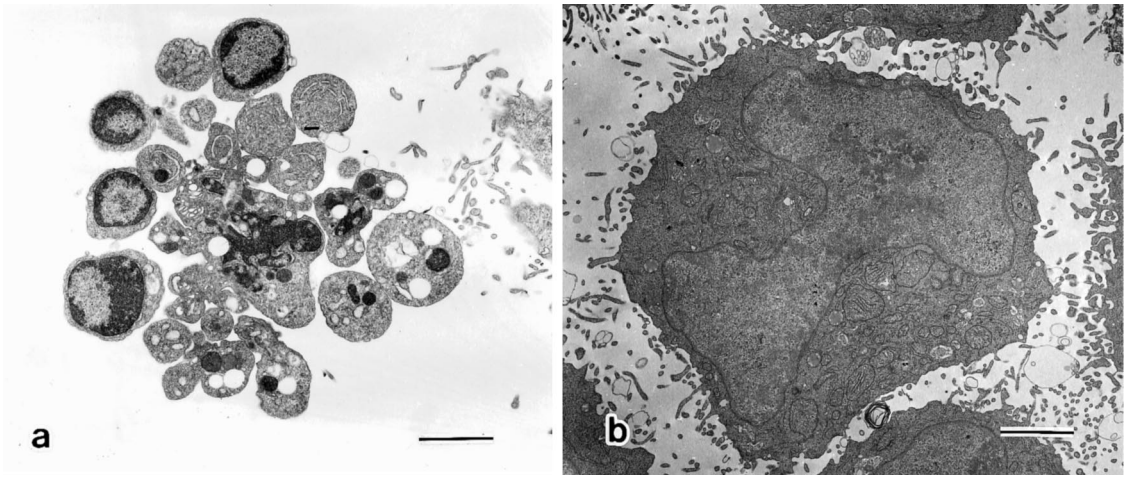


FIG. 1. Electron microscopy of FRTL-5 cells cultured in 0H with (b) or without (a) TSH for 72 h. Note that a typical apoptotic body (a) containing condensed organelle and a half-moon of chromatin contrasts clearly with the control cell (b). 0H, F-12 serum-free medium containing 0.2% BSA without hormones. *Bar*, 2 μm.

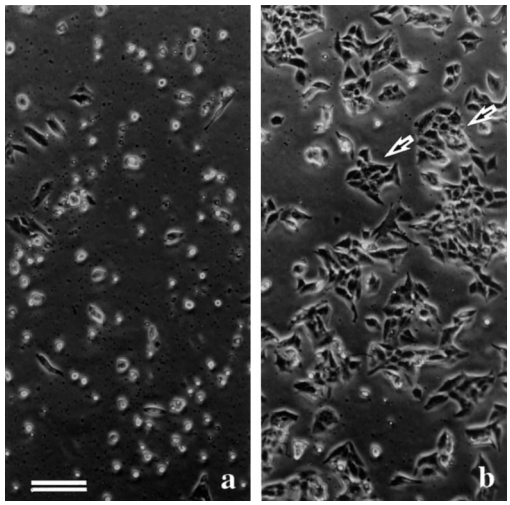


FIG. 2. Phase contrast photomicrograph of FRTL-5 cells cultured in 0H with (b) or without (a) TSH for 72 h. The cells were detached from the dish and floated in the medium in the absence of TSH. In contrast, a tendency to form follicle-like colonies (*arrow* in b) was found in FRTL-5 cells in the presence of TSH. 0H, F-12 serum-free medium containing 0.2% BSA without hormones. *Bar*, 80 μm.

cell viability at 48 h ($p < 0.01$; Fig. 3b). The inhibition was not due to the cytotoxic effect of this inhibitor, as it did not affect the viability of cells cultured in 0H. The above results suggested that TSH might maintain cell viability and prevent apoptosis in FRTL-5 cells mainly via the cAMP/PKA pathway.

The cells in 0H with TSH had intact nuclei (data not shown). However, the cells cultured in 0H without TSH showed extensive nuclear fragmentation after 24 h (*arrow* in Fig. 4a). The fragmented nuclei were positive for TUNEL (*arrow* in Fig. 4b), indicating the presence of nicks in DNA strands of these cell nuclei. Gel electrophoretic analysis of DNA revealed ladders of 180–200 bp in the cells cultured in 0H without TSH. This behavior was not observed in cells cultured in the presence of TSH or cAMP (Fig. 5).

Under an electron microscope, the chromatin of apoptotic cells was either packed into smooth masses against the nuclear membranes, forming half-moon profiles (*arrow* in Fig. 6) or disrupted (*arrowhead* in Fig. 6) in clear contrast to nonapoptotic nuclei (*asterisk* in Fig. 6). Apoptotic bodies were frequently seen. In addition, these cells seemed to have more lysosomes and autophagic activity.

Cell cycle analysis and expression of cell cycle-related proteins in FRTL-5 cells

By flow cytometric analysis, apoptotic cells appeared as the subdiploidy peak before the diploidy peak (*arrows* in Fig. 7a). The percentages of apoptotic cells and the cells in different phases of the cell cycle were derived from the cell cycle analysis software ModFit LTV 1,00. As shown in Fig. 7b, the percentage of apoptotic cells increased time dependently in 0H without TSH. Further, we found that the percentage of cells in the S phase decreased, whereas the cells in the G2/M phase increased simultaneously (Fig. 7a), suggesting that there was cell cycle arrest of the G_1 transition. TSH inhibited the appearance of subdiploidy cells and promoted the progression of the G_1/S phase transition. cAMP showed effects similar to TSH (Fig. 7, a and b).

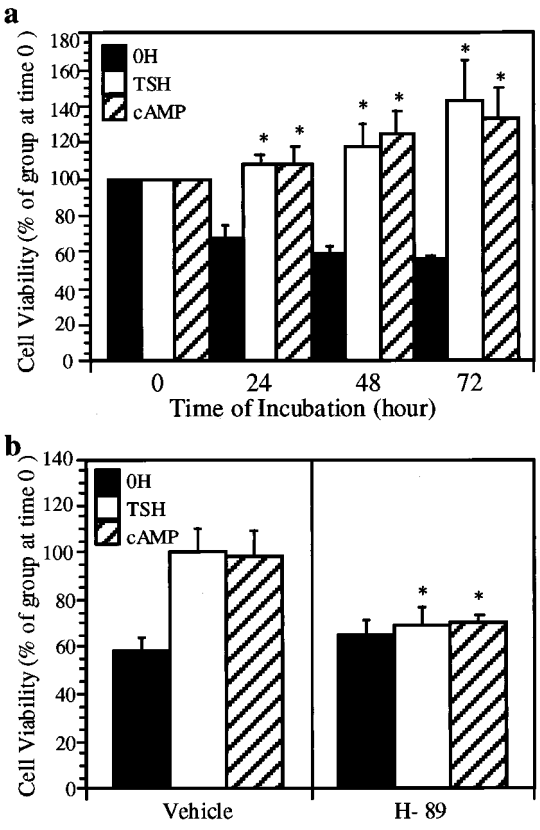


FIG. 3. Evaluation of cell viability by MTT assay. a, Inhibition of apoptosis by TSH or cAMP. FRTL-5 cells were cultured in 0H without or with TSH or cAMP for up to 72 h and processed by MTT assay (see *Materials and Methods*). Cell viability at the indicated times was expressed as the percentage of absorbance of cells at time zero. Data represent the mean ± SD of triplicate determinations from three independent experiments. Statistic analysis was performed using ANOVA followed by Fisher's PLSD. *, $P < 0.0001$ *vs.* 0H. b, Inhibitory effect of H-89 on cAMP-mediated maintenance of cell viability. FRTL-5 cells were pretreated with 25 μM H-89 before stimulation by DMSO vehicle, TSH, or cAMP. Cell viability was evaluated at 48 h as described in a. *, $P < 0.01$, by ANOVA and Fisher's PLSD. 0H, F-12 serum-free medium containing 0.2% BSA without hormones; TSH, 0H plus 10 mU/ml TSH; cAMP, 0H plus 1 mM cAMP.

In an effort to define the mechanism for the changes in cell cycle observed above, we examined the expression of relevant proteins that controlled the G_1-S phase transition by Western blot analysis. The deprivation of serum and hormones resulted in a decreased level of cyclin D expression and overexpression or accumulation of cyclin-dependent kinase inhibitor, p27, after 24-h incubation. TSH increased the expression of cyclin D and decreased the level of p27. cAMP mimicked the TSH effect (Fig. 8), implying that anchorage-dependent FRTL-5 cells cultured in the absence of serum and hormones might activate the pathway of growth arrest and induce apoptosis, and that TSH could prompt the cell cycle progression partly via the cAMP pathway.

Expression of cell adhesion molecules in FRTL-5 cells

To explore the potential mechanisms that might result in these changes in the cell cycle, we examined the expression

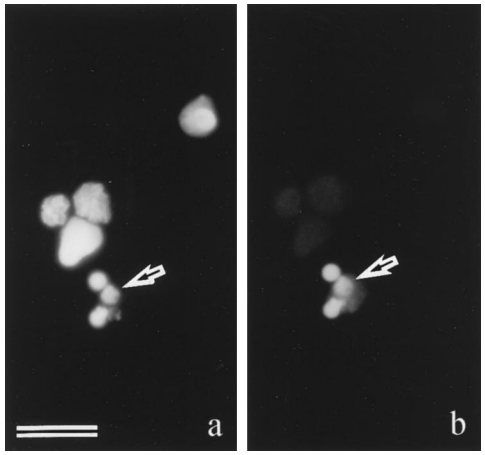


FIG. 4. DNA fragmentation detected by the DNA-binding dye DAPI (a) and the TUNEL method (b). FRTL-5 cells were incubated in 0H without TSH for 72 h to induce apoptosis. The cells were harvested and stained by the TUNEL method and the DNA-binding dye DAPI. The fragmented nucleus (*arrow* in a) was positive for TUNEL (*arrow* in b), whereas nonapoptotic cells were negative. *Bar*, 20 μm.

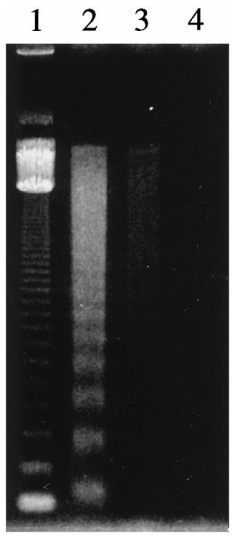


FIG. 5. Agarose gel electrophoresis of DNA from FRTL-5 cells cultured in 0H without (lane 2) or with 10 mU/ml TSH (lane 3) or 1 mM cAMP (lane 4) for 48 h. Lane 1 represents 123-bp DNA mol wt marker of Life Technologies, Inc.

of cell-matrix adhesion molecules, as TSH seemed to promote cell-matrix adhesion as observed by phase contrast microscope. As extracellular matrix proteins, such as fibronectin, laminin, and type IV collagen, have been shown to be survival factors in some cells, we first investigated their effects on the regulation of apoptosis in FRTL-5 cells. Consistent with another report (17), immunofluorescence showed that FRTL-5 cells were stained for the main basement membrane proteins: fibronectin, laminin, and type IV collagen. However, the deprivation of serum and hormones from the medium caused little effect on the expression of these glycoproteins, as revealed by immunofluorescence (Fig. 9). As for the integrin receptors, we observed the expression of α_5 integrin. There was also not a large difference between the groups with and without TSH by immunofluorescence and flow cytometry (data not shown).

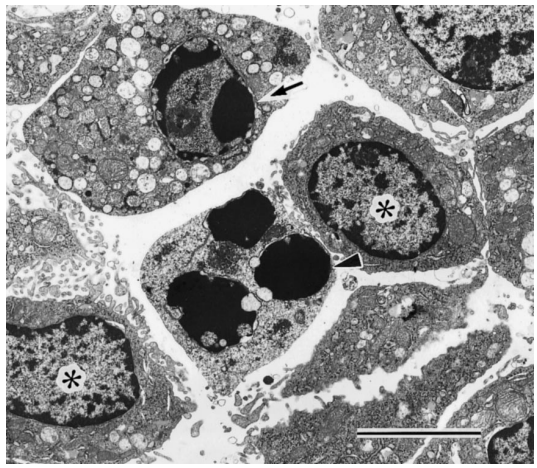


FIG. 6. Electron microscopy of FRTL-5 cells in 0H without TSH for 48 h. The chromatin of some cells was patched into a smooth mass against the nuclear membranes (*arrow*) or disrupted (*arrowhead*), which contrasted with that of nonapoptotic cells (*). *Bar*, 5 μm.

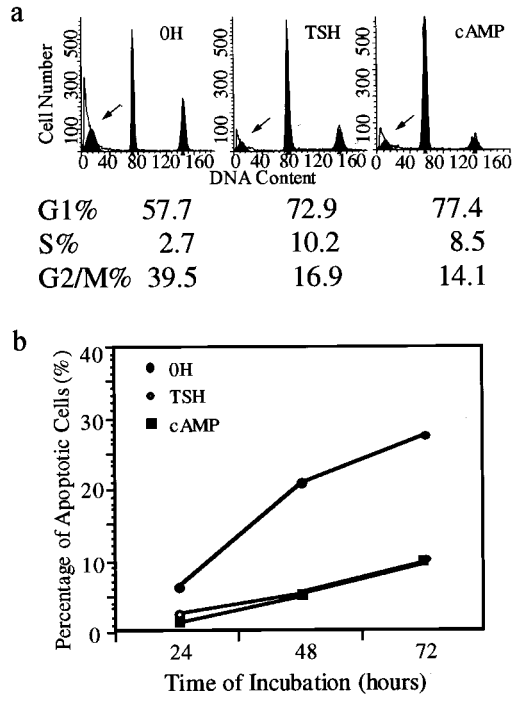


FIG. 7. Flow cytometric analysis of DNA fragmentation and cell cycle. FRTL-5 cells were passaged and cultured in 0H with or without TSH or cAMP for up to 72 h. Then the cells were harvested and stained with PI as described in *Materials and Methods*. The deprivation of serum and hormones resulted in appearance of subdiploidy apoptosis and decreased percentage of cells in S phase (a), implying the arrest of G_1 transition, whereas both TSH and cAMP promoted the progression of the cell cycle (a) and inhibited the apoptosis (a and b). Results showed the representative from three separate experiments. 0H, F-12 serum-free medium containing 0.2% BSA without hormones; TSH, 0H plus 10 mU/ml TSH; cAMP, 0H plus 1 mM cAMP.

Effects of TSH on the expression of FAK and its phosphorylation

To confirm whether the disruption of cell-matrix adhesion triggered apoptosis, we coated the dishes with agarose. The agarose completely prevented cells from attaching to the

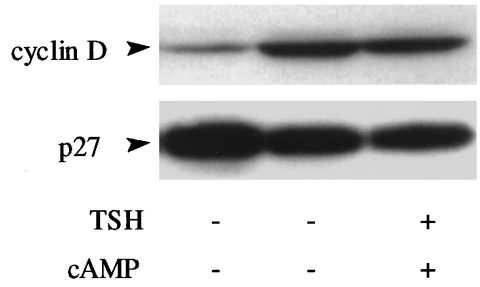


FIG. 8. Western blot analysis of cyclin D and p27 in FRTL-5 cells cultured in 0H with or without TSH or cAMP for 48 h. About 50 μg proteins from cell lysates were run on 12% SDS-PAGE and immunoblotted with a monoclonal antibody against cyclin D. The blots were developed with ECL. After having been deprobed by stripping buffer, the blots were reprobed by a monoclonal antibody against p27 and developed by ECL. The results were confirmed in four separate experiments.

substrate. More than 50% of cells underwent apoptosis even in the presence of TSH (Fig. 10), suggesting that cell-matrix interaction is important for the activation of survival signals. As the cell-matrix interaction is known to stimulate a series of kinases and induce tyrosine phosphorylation of proteins, we then examined the tyrosine phosphorylation of protein of FRTL-5 cell lysates using Western blotting analysis. As shown in Fig. 11, TSH apparently increased the tyrosine phosphorylation of 120- to 125-kDa substrates after 24 h (*arrowhead*). We further examined the expression of FAK and its phosphorylation. TSH was found to increase the expression and phosphorylation of FAK in our culture system (Fig. 12, a and b).

Immunofluorescent staining demonstrated that FAK was localized at the site of cell-matrix adhesion (*green sites* in Figs. 13). We colocalized actin filament with FAK at the contact plaques (*arrowheads* in Fig. 13, a and c) by double staining the FAK-immunolabeled cells with rhodamine-phaloidin (*red sites* in Fig. 13). Similar to another report (18), we also observed the enhanced immunoreactivity of FAK in the cell nuclei (*arrow* in Fig. 13b) and membrane blebs (*arrowhead* in Fig. 13b) in apoptotic cells, but we did not detect fragments from the cleavage of FAK by immunoblot analysis (data not shown).

Discussion

Our initial purpose was to induce apoptosis by the deprivation of serum and hormones in subconfluent FRTL-5 cells. Although we observed some apoptotic cells as evaluated by transmission electron microscope and TUNEL-positive staining, the results of flow cytometry were not satisfactory. In the meantime, we noticed that FRTL-5 cells seemed to gain resistance to apoptosis when they became attached to the substrate. Then we tried to induce apoptosis by deprivation of serum and hormones as soon as subpassage was performed. Under this condition, it was difficult for the FRTL-5 cells to maintain attachment to the substrate, and they underwent apoptosis in the absence of serum and hormones. However, a single addition of TSH from 0.1–100 mU/ml prevented apoptosis (data not shown). It was believed that a low level of calf serum supplement was required for cell attachment for both primary and subsequent passage of FRTL-5 cells and that the presence of the hormones maintained the cultured cell division (7). Here we demonstrated that TSH not only stimulated cell growth, but also promoted cell-matrix adhesion and maintained viability of FRTL-5 cells in the absence of serum and other hormones. Especially during the first 48 h of culture after subpassage, TSH did not result in a significant increase of cell number (data not shown) in the absence of serum and other hormones. In contrast, cell viability was decreased significantly, and apoptosis occurred in the absence of TSH. Thereafter, possibly because of the mitogenic effect of TSH, cell viability increased greatly, and cell number also began to increase. Thus, cell-matrix adhesion and cell survival seem to be essential for the subsequent growth of FRTL-5 cells.

As early as 1978, Folkman and Moscona proposed that appropriate cell shape was critical for DNA synthesis by normal cells, which was explained as the theory of anchorage dependence (19). This means that normal cells will not grow unless attached to a substratum. When the interaction between normal cells and the extracellular matrix is blocked, apoptosis occurs. This phenomenon was named anoikis, a Greek word meaning homelessness, by Frisch and Francis (20). Anoikis was first reported by Meredith *et al.* (21), who induced apoptosis by incubating human umbilical vein endothelial cells by plating the cells on agarose-coated dishes or in the absence of serum and growth factors. In addition to endothelial cells, anchorage-dependent apoptosis was also observed in kidney cells (18), mammary cells (22), ovarian cells (23, 24), melanocytes (25), and oncogene-transformed fibroblasts (26). To our knowledge, this is the first report of anchorage-dependent apoptosis induced in thyroid cells.

Extracellular matrix (ECM) proteins such as fibronectin, laminin, and type IV collagen have been considered to be survival factors for many cell types. Cell-ECM adhesion is mainly involved in the interaction of integrins and these extracellular proteins. The downstream signaling events may associate or activate a number of nonreceptor protein tyrosine kinases, including FAK, which might provide the cell survival signal or facilitate gene expression and cell cycle progression events (27). Consistent with another report (17), we demonstrated that FRTL-5 cells synthesized and secreted the main ECM proteins. On the other hand, thyroid cells were reported to express integrins such as $\alpha_1\beta_1$, $\alpha_3\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$ (28). α_5 integrin was localized in FRTL-5 cells by immunofluorescence in our study (data not shown). As apoptosis still occurred despite the binding of ECM to integrin receptors when TSH and serum were absent, we hypothesized that the simple ligation of integrin was not sufficient for the maintenance of viability of FRTL-5 cells under these conditions. Other events that are activated by TSH may be needed. FAK, which is thought to act as an integrin-mediated signal transducer, was reported to play a role in the prevention of apoptosis in diverse cell types (29–31). Inhibition of FAK resulted in apoptosis in fibroblasts (29), and its overexpression prevented anoikis (30, 31). Immunoblot analysis in our study demonstrated that TSH increased the expression of phosphorylated FAK, suggesting that FAK may also be involved in the maintenance of TSH-dependent survival in FRTL-5 cells.

Protein tyrosine phosphorylation was found to regulate

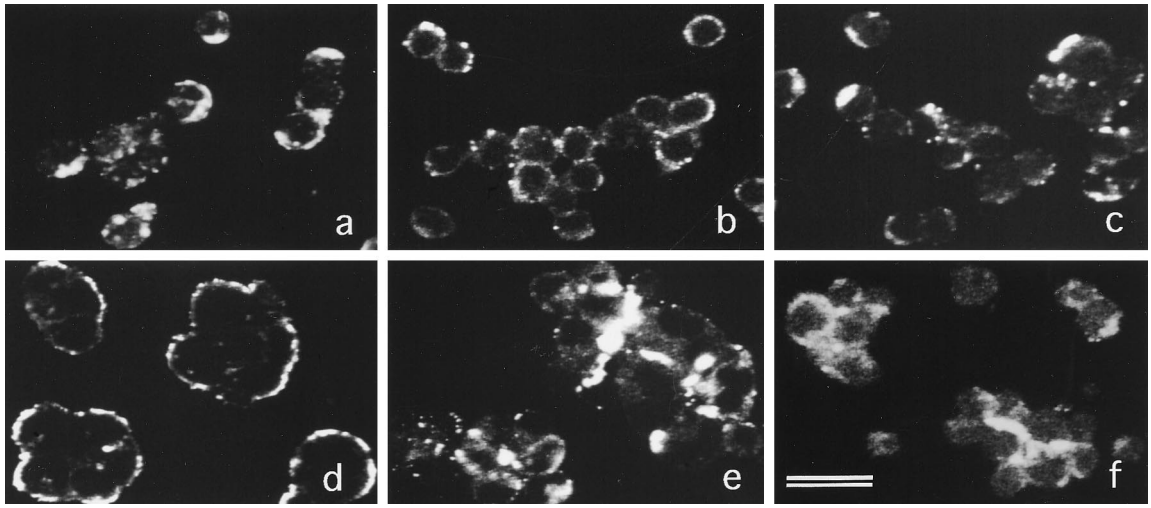
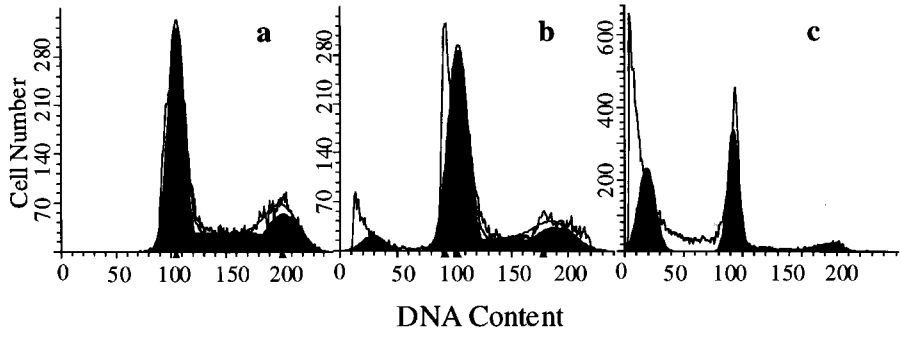


FIG. 9. Immunofluorescent staining of FRTL-5 cells for fibronectin (a and d), laminin (b and e), and type IV collagen (c and f). FRTL-5 cells were passaged onto glass coverslips and cultured in 0H with (d–f) or without (a–c) TSH for 48 h. The deprivation of TSH caused little effect on the expression of these ECM proteins. *Bar*, 20 μm.

FIG. 10. Effect of disruption of cell-matrix interaction on apoptosis evaluated by PI-stained flow cytometry. FRTL-5 cells were cultured on agarose-coated (c) or uncoated dishes (b) in the presence of TSH. The cells were harvested at 0 h (a) or after 72 h (b and c) and processed as described in Fig. 7. The blockage of cell-matrix interaction resulted in apoptosis in FRTL-5 cells even in the presence of TSH.



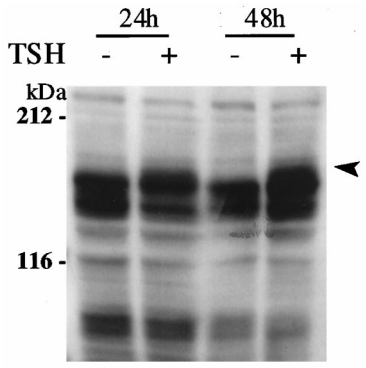


FIG. 11. Western blot analysis of tyrosine phosphorylation from whole cell lysates of FRTL-5 cells cultured in 0H with or without TSH for the indicated times. The proteins were extracted, quantified, and separated using 8% SDS-PAGE. Then the proteins were transferred and immunoblotted using antiphosphotyrosine antibodies (4G10 and PY20). TSH increased the tyrosine phosphorylation of 120- to 125-kDa substrate after 24 h (*arrowhead*). Similar results were obtained in three separate experiments.

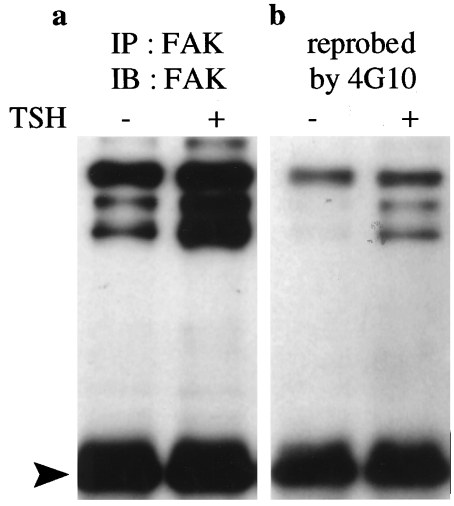


FIG. 12. Expression of FAK (a) and its tyrosine phosphorylation (b) in FRTL-5 cells cultured in 0H with or without TSH for 24 h. The cells were lysed and immunoprecipitated with an anti-FAK monoclonal antibody. The immunoprecipitates were resolved by 8% SDS-PAGE and transferred onto a membrane, and the blots were probed with another anti-FAK antibody (a). After development by ECL, the membrane was deprobed by stripping buffer and reprobed by antiphosphotyrosine antibodies (4G10 and PY20; b). TSH up-regulated the expression of FAK and its phosphorylation. The lowest bands (*arrowhead*) were heavy chains of Ig.

the TSH-induced increase in proliferation and iodide uptake through the cAMP pathway in FRTL-5 cells (32). While exploring the effect of TSH on the regulation of growth in FRTL-5 cells, Takahashi *et al.* (33) also demonstrated that TSH potentiated IGF-I-dependent tyrosine phosphorylation of 175-kDa substrate and stimulated IGF-I-independent accu-

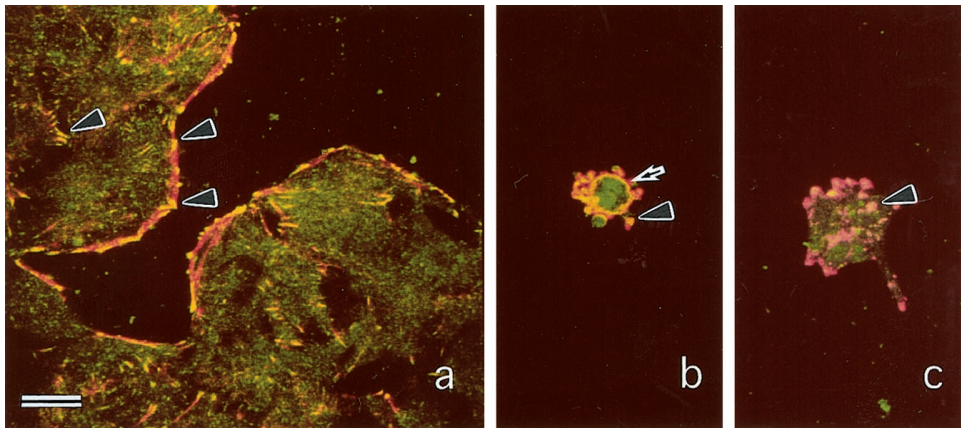


FIG. 13. Double staining of FAK and actin filament in FRTL-5 cells by immunofluorescence. FRTL-5 cells were passaged onto coverslips and cultured in 6H5 (a) or 0H with (c) or without (b) TSH for 48 h. Then the cells were fixed with 4% paraformaldehyde, permeabilized, and immunostained with an anti-FAK antibody, followed by double staining for actin filament using rhodamine-phalloidin. FAK was labeled as *green* fluorescence due to the fluorescein isothiocyanate-labeled secondary antibody. Actin filament was demonstrated as *red* fluorescence due to the rhodamine-labeled phalloidin. Colocalization appeared in *yellow* under two channels of TRITC/fluorescein isothiocyanate by a confocal scanning microscope. FAK and actin filament were colocalized at the sites of contact plaques (*arrowheads* in a and c). There was enhanced immunoreactivity in the nuclei (*arrow* in b) and membrane blebs (*arrowhead* in b) in apoptotic cells. *Bar*, 15 μm.

mulation of tyrosine phosphorylation. The latter TSH-dependent phosphorylation was largely confined to the band of 120–125 kDa. The 175-kDa substrate was believed to be related to cytoskeletal proteins, whereas the properties of the 120- to 125-kDa substrate were not determined. Our findings obtained from Western blot analysis of whole cell lysates by antityrosine phosphorylation antibody also demonstrated that TSH strengthened or probably stimulated the tyrosine phosphorylation of 120–125 kDa substrate. The result of immunoprecipitation with anti-FAK antibody showed increased expression of FAK and its tyrosine phosphorylation after TSH stimulation, suggesting that at least FAK may be included in this band.

Recent studies have established the role of cell cycle relevant proteins in the regulation of apoptosis (34, 35). A cyclin-dependent kinase inhibitor, p27, was reported to result in cell cycle arrest of the G_1 to S phase transition by associating with cyclin D-CDK4 complex and potentially inhibiting Rb phosphorylation (36). Overexpression of p27 resulted in the G_1-S arrest and triggered apoptosis in cancer cells (34). Recently, Carneiro *et al.* reported the induction of apoptosis in FRTL-5 cells by transforming growth factor-β1 in the absence of TSH (10), accompanied by increased expression of p15 and c-*myc* messenger RNA and decreased expression of cyclin D1 and P27. Apoptosis induced in our study was accompanied by decreased expression of cyclin D without a decreased level of p27, but even with a slightly increased expression of p27. These changes were coincident with the onset of apoptosis (*i.e.* after 24 h; data not shown). Similarly, recent papers using FRTL-5 cells also demonstrated the relation of p27 accumulation to the arrest of G_1/S transition (37, 38), in which degradation of p27 was induced during G_1/S transition by growth stimulation, including calf serum, insulin, and TSH. In the present study, we showed that TSH alone had apparent effects on the cell cycle-relevant proteins in FRTL-5 cells.

Several pieces of evidence indicated that cross-talk may exist between the pathway of cell adhesion and cell cycle regulation (27, 39–41). The cyclin E-CDK2 complex was activated in late G_1 phase in attached human fibroblasts, but not in suspension-cultured cells. This decreased cyclin E-CDK2 activity in suspension cells resulted from an increase in the amount of CDK2 inhibitors, such as p27 and p21 (39). Also, forced expression of exogenous cyclin A made NRK fibroblasts partially independent of anchorage (40). These data imply a dependence of cyclin/cyclin-dependent kinase activity on cell anchorage. More recently, overexpression of wild-type FAK was reported to accelerate the G_1 to S phase transition by increasing cyclin D1 expression and decreasing p21 expression (41). Our results also suggested that increased expression of phosphorylated FAK could be involved in the promotion of cell cycle progression in FRTL-5 cells stimulated by TSH. The factors residing between the proximal cell adhesion events and distal cell cycle machinery were not clear. Lately, the Rho family of small guanosine triphosphatases, which includes Rho, Rac, and Cdc42, was reported to be a molecular switch controlling a signal transduction pathway that links membrane receptors to gene transcription (42). Rho-related kinases exert their biological functions in the rearrangement of the cellular skeleton and enhancement of focal adhesion (43, 44). In addition, the Rho family plays a pivotal role in G_1 progression and DNA synthesis in response to mitogenic stimulator (45). It was shown that the geranylation of Rho A protein and its translocation to membranes were essential for the degradation of p27 and G_1/S transition in growth-stimulated FRTL-5 cells (37, 38). The effect of the Rho family on regulating apoptosis remains to be studied.

In conclusion, TSH prevents apoptosis by increasing cell-matrix adhesion and facilitating cell cycle progression partly via the cAMP pathway in FRTL-5 cells, thus suggesting that in addition to regulating growth and differentiation, TSH may function as a survival factor in thyroid cells.

References

1. **Aoki MP, Maldonado CA, Aoki A** 1998 Apoptotic and non-apoptotic cell death in hormone-dependent glands. Cell Tissue Res 291:571–574
2. **Furuya Y, Isawa JT, Shimazaki J** 1995 Induction of programmed death/apoptosis in androgen-dependent mouse mammary tumor cell line by androgen withdrawal. Jpn J Cancer Res 86:1159–1165
3. **Tilly JL, Tilly KI** 1995 Inhibitors of oxidative stress mimic the ability of follicle stimulating hormone to suppress apoptosis in cultured rat ovarian follicles. Endocrinology 136:242–252
4. **Tanimoto C, Hirakawa H, Kawasaki H, Hayakawa N, Ota Z** 1995 Apoptosis in thyroid diseases–a histochemical study. Endocr J 42:193–201
5. **Dremier S, Gelsten J, Mosselmans R, Domont JE, Galand P, Rubaye B** 1995 Apoptosis in dog thyroid cells. Biochem Biophys Res Commun 200:52–58
6. **Kawakami A, Eguchi K, Matsuoka N** 1996 Thyroid stimulating hormone inhibits Fas antigen-mediated apoptosis of human thyrocytes *in vitro*. Endocrinology 137:3163–3169
7. **Ambesi-Impiombato FS, Parks LAM, Coon HG** 1980 Culture of hormone dependent functional epithelial cells from rat thyroid. Proc Natl Acad Sci USA 77:3455–3459
8. **Ambesi-Impiombato FS, Villone G** 1987 The FRTL-5 thyroid cells strains as a model for studies on thyroid cell growth. Acta Endocrinol [Suppl] 281:242–245
9. **Nitsch L, Garbi C, Gentile R, Masica A, Negri R, Polistina C, Vergani G, Zurzolo C** 1990 Morphological changes induced by prolonged TSH stimulation or starvation in the rat thyroid cell line FRTL. Horm Metab Res [Suppl] 7:32–37
10. **Carneiro C, Alvarez CV, Zalvide J, Vidal A, Dominguez F** 1998 TGF-β1 actions on FRTL-5 cells provide a model for the physiological regulation of thyroid growth. Oncogene 16:1455–1465
11. **Ginsberg J, Gupta S, Matowe WC, Kline L, Brindley DN** 1997 Activation of phospholipase P in FRTL-5 thyroid cells by forskolin and dibutyryl-cyclic adenosine. Endocrinology 138:3645–3651
12. **Gavrieli Y, Sherman Y, Ben-Sasson SA** 1992 Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 119:493–501
13. **Bissonnette RP, Echeverri F, Mahboubi A, Green DR** 1992 Apoptotic cell death induced by c-*myc* is inhibited by *bcl*-2. Nature 359:552–554
14. **Nicoletti I, Migliorati G, Pagliacci F, Riccardi** 1991 A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 139:271–279
15. **Ealey PA, Ahene CA, Emmerson JM, Marshall NJ** 1987 Forskolin and thyrotrophin stimulation of rat FRTL-5 thyroid cell growth: the role of cyclic AMP. J Endocrinol 114:199–205
16. **Valente WA, Vitti P, Kohn LD, Brandi ML, Rotella CM, Touafondi R, Tramontano D, Aloj SM, Ambesi-Impiombato FS** 1983 The relationship of growth and adenylate cyclase activity in cultured thyroid cells: separate bioeffects of thyrotropin. Endocrinology 112:71–79
17. **Garbi C, Zurzolo C, Bifuleo M, Nitsch L** 1988 Synthesis of extracellular matrix glycoproteins by a differential thyroid epithelial cell line. J Cell Physiol 135:39–46
18. **Levkau B, Herren B, Koyama H, Ross R, Raines E** 1998 Caspase-mediated cleavage of focal adhesion kinase pp125 FAK and disassembly of focal adhesions in human endothelial cell apoptosis. J Exp Med 187:579–586
19. **Folkman J, Moscona A** 1978 Role of cell shape in growth control. Nature 273:345–349
20. **Frisch SM, Francis H** 1994 Disruption of epithelial cell-matrix interaction induces apoptosis. J Cell Biol 124:619–626
21. **Meredith JE, Fazeli B, Schwartz MA** 1993 The extracellular matrix as a cell survival factor. Mol Biol Cell 4:953–961
22. **Boudreau N, Sympson CJ, Werb Z, Bissel MJ** 1995 Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. Science 267:891–893
23. **Zhang Z, Vuori K, Reed JC, Rouslahti E** 1995 The $\alpha_5\beta_1$ integrin supports survival of cells on fibronectin and up-regulates *bcl-2* expression. Proc Natl Acad Sci USA 92:6161–6165
24. **Aharoni D, Meiri I, Atzmon, Vlodarsky I, Amsterdam A** 1996 Differential effect of components of the extracellular matrix on differentiation and apoptosis. Curr Biol 7:43–51
25. **Scott G, Cassidy L, Busacco A** 1997 Fibronectin suppresses apoptosis in normal human melanocytes through an integrin-dependent mechanism. J Invest Dermatol 108:147–153
26. **McGill G, Shimamura A, Bates RC, Savage RE, Fisher DE** 1997 Loss of matrix adhesion triggers rapid transformed-selective apoptosis in fibroblasts. J Cell Biol 138:901–911
27. **Schlaepfer DD, Hunter T** 1998 Integrin signaling and tyrosine phosphorylation: just the FAKs. Trends Cell Biol 8:151–157
28. **Vitale M, Bassi V, Fenzi G, Mauhia PE, Salzano S, Rossi G** 1993 Integrin expression in thyroid cells from normal glands and nodular goiters. J Clin Endocrinol Metab 76:1575–1579
29. **Hungerford JE, Compton MT, Matter ML, Hoffstrom BG, Otey CA** 1996 Inhibition of pp125 FAK in cultured fibroblasts results in apoptosis. J Cell Biol 135:1383–1390
30. **Frisch SM, Vuori K, Ruoslahti E, Chan-Hui PY** 1996 Control of adhesion dependent cell survival by focal adhesion kinase. J Cell Biol 134:793–799
31. **Zheng C, Xing Z, Bian ZC, Guo C, Akbay A, Warner L, Guan JL** 1998 Differential regulation of pyk2 and focal adhesion kinase (FAK). J Biol Chem 273:2384–2389
32. **Mori K, Stone S, Braverman LE, Devito WJ** 1996 Involvement of tyrosine phosphorylation in the regulation of 5′-deiodinases in FRTL-5 rat thyroid cells and rat astrocytes. Endocrinology 137:1313–1318
33. **Takahashi SI, Conti M, Prokop C, Van Wyk JJ, Earp HS** 1991 Thyrotropin and insulin-like growth factor regulation of tyrosine phosphorylation in FRTL5 cells. J Biol Chem 266:7834–7841
34. **Katayose Y, Kim M, Rakkar A, Li Z, Cowan KH, Seth P** 1997 Promoting apoptosis: a novel activity associated with the cyclin dependent kinase inhibitor P27. Cancer Res 57:5441–5445
35. **Bos C, Silverstetter S, Marphy M, Connolly T** 1998 $P21^{cip1}$ rescues human mesenchymal stem cells from apoptosis induced by low-density culture. Cell Tissue Res 293:463–47031
36. **Polyak K, Lee MH, Erdjument-Bromage H, Koff A, Roberts M, Tempst P** 1994 Cloning of $p27^{kip1}$, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell 78:59–66
37. **Hirai A, Nakamura S, Noguchi Y, Yasuda T, Kitagawa M, Tatsuno I,Oeda T, Tahara K, Terano T, Narumiya S, Kohn LD, Saito Y** 1997 Geranylgeranylated rho small GTPase(s) are essential for the degradation of $p27^{kip1}$ and facilitate the progression from G1 to S phase in growth stimulated rat FRTL-5 cell. J Biol Chem 272:13–16
38. **Noguchi Y, Nakamura S, Yasuda T, Kitagawa M, Kohn LD, Saito Y, Hirai A** 1998 Newly synthesized Rho A, not Ras, is isoprenylated and translocated to membranes coincident with progression of the G1 to S phase of growth-stimulated rat FRTL-5 cells. J Biol Chem 273:3649–3653
39. **Fang F, Orend G, Watanabe N, Hunter T, Ruoslahti E** 1996 Dependence of cyclin E-CDK2 kinase activity on cell anchorage. Science 271:499–502
40. **Guadagno TM, Ohtsubo M, Roberts JM, Assoian RK** 1993 A link between cyclin A expression and adhesion-dependent cell cycle progression. Science 262:1572–1575
41. **Zhao J, Reiske H, Guan J** 1998 Regulation of the cell cycle by focal adhesion kinase. J Cell Biol 143:1997–2008
42. **Hall Alan** 1998 Rho GTPase and the actin cytoskeleton. Science 279:509–514
43. **Chihara K, Amano M, Nakamura N, Yano T, Shibata M, Tokui T, Ichikawa H, Ikebe R, Ikebe M, Kaibuchi K** 1997 Cytoskeletal rearrangements and transcriptional activation of c-Fos serum response element by Rho-kinase. J Biol Chem 272:25121–25127
44. **Amano M, Chihara K, Kimura K, Fukata Y, Nakamura N, Matsuura Y, Kaibuchi K** 1997 Formation of actin stress fibers and focal adhesions enhanced by Rho-kinase. Science 275:1308–1311
45. **Olson MF, Ashworth A, Hall A** 1995 An essential role for Rho, Rac, and Cdc42 GTPases in cell cycle progression through G1. Science 269:1270–1272