0021-972X/00/$03.00/0
The Journal of Clinical Endocrinology & Metabolism

Vol. 85, No. 3
Printed in U.S.A.

Serum Withdrawal-Induced Apoptosis in Thyroid Cells Is Caused by Loss of Fibronectin-Integrin Interaction*

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ABSTRACT

In some cell types, including a fetal thyroid cell line, denial of adhesion to extracellular matrix induces a type of apoptosis called anoikis. Serum withdrawal in dog and transformed rat thyroid cells also induces programmed cell death. Because serum can stimulate cells to produce some components of the extracellular matrix, it was of interest to determine the role of the matrix in the apoptosis induced by serum withdrawal in normal human thyroid cells in primary culture. The present report demonstrates that thyroid cells selectively produce and deposit insoluble fibronectin (FN) only when stimulated by serum. Adhesion in the presence of serum is dependent upon integrin-FN interaction. Serum withdrawal determines a degradation of the insoluble FN deposited and a detachment of the cells from the plates. In these conditions, cells undergo anoikis, demonstrated by DNA fragmentation and annexin V staining. Apoptosis was prevented by exogenous FN immobilized onto the plates. These results indicate that serum withdrawal induces apoptosis in human thyroid cells, determining FN degradation and loss of cell-matrix adhesion. (*J Clin Endocrinol Metab* **85:** 1188–1193, 2000)

APOPTOSIS, OR programmed cell death, can be triggered by both physiological and pathological stimuli, such as cytokines (1–3), growth factors, radiations, and anticancer drugs (4). Hormone-dependent tissues, such as the prostate and mammary glands (5, 6) or uterine epithelium, undergo apoptosis after hormone depletion. Recent studies demonstrate that extracellular matrix (ECM), such as hormones and grow factors, in addition to regulating cell growth and differentiation, is also a survival factor for many cell types (7, 8). The ECM of the basement membrane induces differentiation and regulates cell survival, suppressing apoptosis in different endothelial and epithelial cell types (9). Involution of hormone-dependent tissues displays the linkage existing between growth factor deprivation and apoptosis induced by ECM degradation. A prototype of such linkage is the mammary epithelial cells that, lacking their laminin-rich basement membrane during estrogen deprivation, lose their differentiated phenotype, and undergo apoptosis (6). Although the molecular mechanisms of this survival effect is poorly understood, it is clear that integrin activation plays a central role in coordinating the expression of positive and negative regulators of apoptosis. Integrins and growth factors share many intracellular signaling pathways controlling cellular functions, such as tyrosine phosphorylation, inositol lipid metabolism, and Ca^{2+} fluctuation.

Serum withdrawal in endothelial cells as well as in canine thyroid primary cultures and transformed rat thyroid cells also induces programmed cell death (10, 11). Serum can be considered for many cell types a complex mixture of growth factors that regulate several cellular functions, including, in certain cell types, the synthesis of ECM, and have important effects on the cellular environment. Follicular cells in the thyroid are surrounded by a continuous rim of matrix proteins whose major components are fibronectin (FN) and laminins (12). Thyrocytes can interact with the FN of the basement membrane through the integrin receptors $\alpha_3\beta_1$ and $\alpha_v\beta_3$, largely expressed on the cell membrane (13–15). Thus, basement membrane-integrin interactions can have major effects on the phenotypic features of thyroid cells controlling growth, differentiation, and hormonal response.

In this study we show that FN production induced by serum is required for the survival of human thyroid cells in primary culture. Our results indicate that apoptosis induced by serum withdrawal is caused by ECM degradation and loss of normal integrin-FN interactions.

Materials and Methods

Cell cultures

Tissue specimens were obtained at surgery from internodular tissue of nodular goiters undergoing thyroidectomy. Cell cultures were prepared as previously described (16, 17). Briefly, tissues were chopped and digested by type IV collagenase (Sigma, St. Louis, MO; 1.25 mg/mL) in Ham's F-12 medium and 0.5% BSA overnight at 4 C under rotation. Cells were pelleted by centrifugation at 150 × *g* for 5 min, washed twice in BSA-Ham's F-12 medium (BSA/F-12), seeded in petri dishes, and cultured in a 5% CO_2 atmosphere at 37 C in Ham's F-12 medium supplemented with 10% FCS. Where indicated, bovine TSH (bTSH) purchased from Sigma was used. Medium was changed every 3–4 days, and all cells used in the experiments were harvested from 3- to 7-day-old cultures.

Received July 27, 1999. Revision received October 12, 1999. Accepted November 11, 1999.

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* This work was supported by Ministero dell'Universitá e della Ricerca Scientifica and Associatione Italiana per la Ricerca sue Cancer.

The follicular origin of the cells (>95%) was confirmed by flow cytofluorometry using anticytokeratin and thyroglobulin antibodies as previously described (14).

Immunofluorescence

Cells were plated onto sterile glass coverslips and cultured for up to 72 h at 37 C in F-12 and 10% FCS. Cells were rinsed in phosphate-buffered saline (PBS), fixed in 70% ethanol PBS for 10 min, and blocked in 0.5% BSA for 10 min. Cells were incubated with rabbit serum antihuman FN or collagen I (CoGI; Chemicon, Temecula, CA) in PBS and 0.2% Tween-20 for 1 h, washed in PBS, incubated with fluorescein-conjugated secondary antibody for 30 min, washed again, briefly rinsed in distilled water, mounted on microscope slides in PBS and 50% glycerol, and observed with a fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

Enzyme-linked immunoassay

A total of 1.5×10^4 cells/well were plated in 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA) in F-12 medium with or without serum and cultured. Then, cells were fixed by methanol-acetone (vol/vol) for 10 min at room temperature and air-dried. Wells were filled with 100 μL 2% rabbit serum antihuman FN in PBS, 0.5% BSA, and 0.2% Tween-20 and allowed to react for 1 h at 4 C. Then the plates were washed with PBS, filled with 100 μL horseradish peroxidase-conjugated antirabbit IgG in PBS-0.2% Tween-20, allowed to react for 1 h, washed with PBS, and filled with 150 μL 1 mg/mL *o*-phenylenediamine, 0.006% hydrogen peroxide, and 0.1 mol/L citrate buffer, pH 5.0. After 30-min incubation, the absorbance at 450 nm was measured by a spectrophotometer. Rabbit serum antihuman FN did not react with bovine FN.

Cell attachment assay

A total of 2×10^4 cells/well were seeded in microtiter plates in F-12 medium and 10% FCS and cultured for 6 h. Where indicated, cells were coincubated with 100 μg/mL antihuman FN, RGD-containing peptides (RGSP = Gly-Arg-Gly-Asp-Ser-Pro; RGTP = Gly-Arg-Gly-Asp-Thr-Pro), or RGE-containing peptides (Gly-Arg-Gly-Glu-Ser-Pro) (Telios). Then, the plates were gently washed with PBS, and the attached cells were fixed with 3% paraformaldehyde for 10 min followed by 2% methanol for 10 min and finally stained with 0.5% crystal violet in 20% methanol. After 10 min, the plates were washed with tap water, the stain was eluted with a solution of 0.1 mol/L sodium citrate, pH 4.2, in 50% ethanol, and the absorbance at 540 nm was measured by a spectrophotometer. All of the experiments were performed in quadruplicate. Results were expressed as a percentage of the adhesion obtained in the absence of peptides.

DNA electrophoresis and estimation of apoptotic cells

Suspended cells collected by centrifugation and adherent cells were washed in PBS; lysed in 300 μL 0.5% Triton X-100, 5 mmol/L Tris-buffer (pH 7.4), and 20 mmol/L ethylenediamine tetraacetate for 20 min at 4 C; and centrifuged at 13,000 rpm for 30 min. Centrifugation-resistant low molecular weight DNA was extracted with phenol/chloroform, precipitated with ethanol, and incubated with 0.5 μg/mL ribonuclease A, deoxyribonuclease-free for 30 min at 37 C. DNA with loading buffer were electrophoresed in 1% agarose and 1 μg/mL bromide at 50 V in 45 mmol/L Tris-borate and visualized by UV.

Annexin V assay for determination of apoptosis/necrosis ratio was performed as follows. Cells were washed twice with cold PBS; resuspended in 10 mmol/L HEPES (pH 7.4), 140 mmol/L NaCl, and 2.5 mmol/L $CaCl_2$; and incubated for 15 min at room temperature with fluorescein-conjugated annexin V (PharMingen) and 5 μg/mL propidium iodide. Cells were analyzed within 1 h by flow cytometry using a FACScan (Becton Dickinson and Co., Mountain View, CA).

Cytofluorimetric estimation of apoptosis was performed as follows: floating cells were collected, washed in cold PBS, added to adherent cells, and trypsinized. Cells were washed again in PBS and fixed in 70% cold ethanol for 30 min. Ethanol was removed by two PBS washes, and cells were incubated in PBS, 50 μg/mL propidium iodide, and 10 μg/mL ribonuclease A, deoxyribonuclease-free overnight at 4 C. Cells were then analyzed by flow cytometry using a FACScan.

Results

Cultured thyroid cells stimulated by serum produce FN

To determine whether normal thyroid cells produce ECM in culture, cells harvested from 3- to 7-day-old primary cultures were plated in F-12 medium and 0.2% BSA with or without 10% FCS or without FCS in the presence of 10 μg/mL soluble FN. After 6, 12, 24, and 48 h, medium was removed, and adherent cells and insoluble matrix were fixed. The cells were observed with a phase contrast microscope, and the presence of FN and CoGI was determined by enzyme-liked immunoassay with specific antisera (Fig. 1). In the presence of FCS, cells attached and spread, acquiring a flat polygonal shape after 1 h. FN was detectable in 6-h cultures and progressively increased with time, demonstrating that cells synthesize and deposit insoluble FN. In the absence of FCS, with or without soluble FN, cells were adherent, but retained a round shape after 1 h, then a modest spreading was observed, and cells acquired a spindle shape. A small amount of FN that remained constant during the culture was detected by enzyme-liked immunoassay in the following hours. Treatment with 10% FCS after 48-h culture without serum rapidly stimulated the cells to produce FN. CoGI was not detected in the presence of FCS or in its absence.

Cells were plated in F-12 medium and 10% FCS on glass coverslips and cultured for 72 h. Complete cell spreading and actin microfilament organization required several hours to occur. Cytoskeletal organization, cell spreading, and deposition of ECM fibrils were progressive and proceeded in parallel (not shown). After 72 h of culture, a dense net of FN fibrils was deposited by the cells, whereas CoGI was not detected (Fig. 2). The effect of bTSH (0.5–10 mU/mL) alone and in combination with FCS was also investigated. After 72 h of culture in the presence of FCS and 10 mU/mL bTSH, the cells displayed a less flat morphology (not shown), and insoluble FN deposition was only slightly increased (Fig. 3),

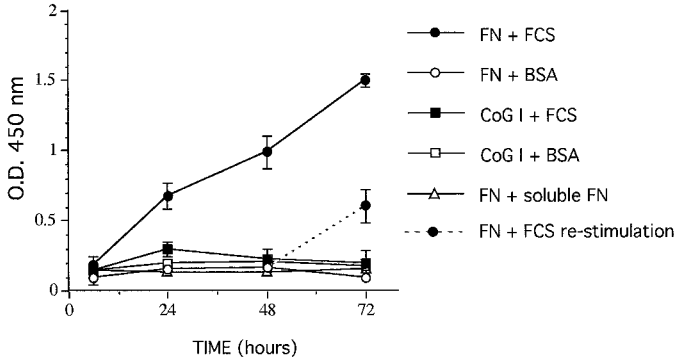

FIG. 1. Thyroid cells deposit FN, but not CoGI. Normal thyroid cells from 7-day-old primary cultures were cultured in 10% FCS containing medium (*solid symbols*) or 0.2% BSA (*open symbols*) alone or with 10 μg/mL soluble FN (*triangles*). In a parallel experiment, 10% FCS was added after 48 h of culture in serum-free medium (*dotted line*). At appropriate times, the presence of FN (*circles*) or GoG I (*squares*) was estimated by enzyme-linked immunoassay. Relative ECM content per well is expressed as the mean absorbance ± SD of triplicate wells.

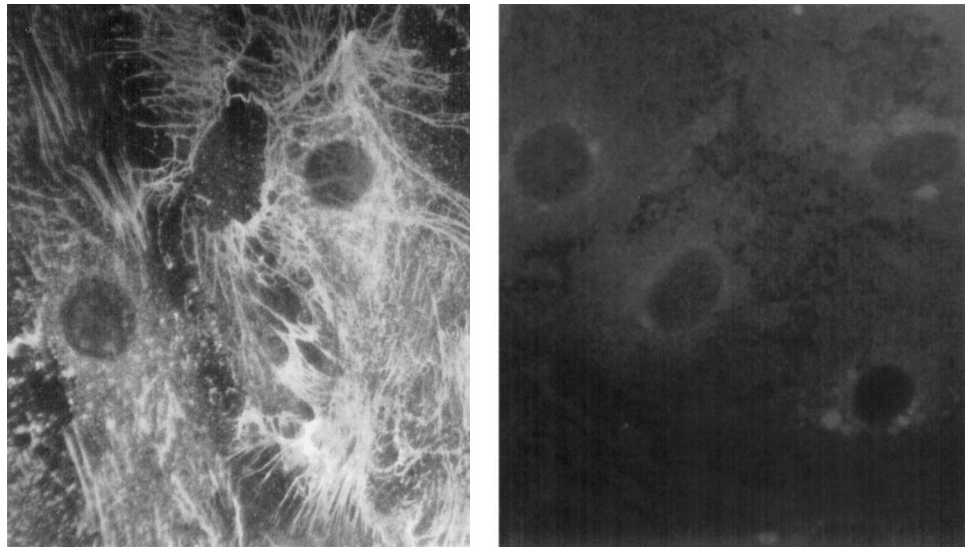

FIG. 2. Deposition of insoluble ECM by thyroid cells in culture. Normal thyroid cells were plated in 10% FCS containing medium on coverslips, and after 72 h of culture the matrix was stained by indirect immunofluorescence. Insoluble FN fibrils (*left*) were deposited on the coverslips, but not CoGI (*right*).

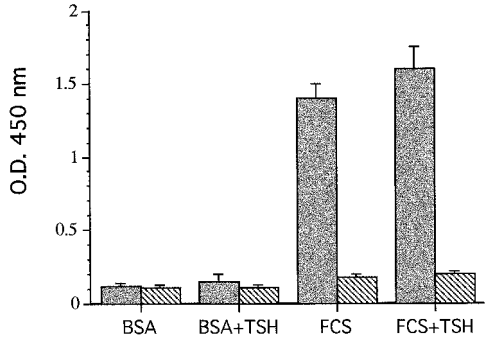

FIG. 3. Deposition of insoluble ECM upon TSH treatment. FN (*solid bars*) and CoGI (*slashed bars*) productions were estimated by enzyme-linked immunoassay after 72 h of culture in the presence of TSH alone (BSA+TSH) or in combination with FCS (FCS+TSH).

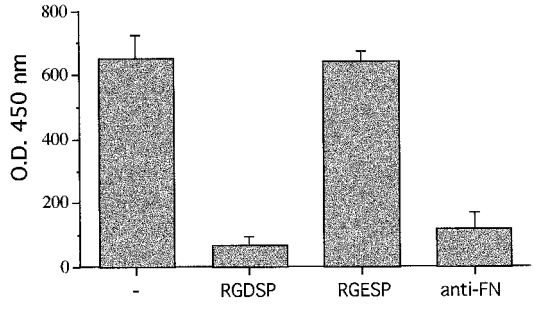

FIG. 4. Inhibition of thyroid cell adhesion in serum-containing cultures. Normal thyroid cells were plated in medium and 1% FCS in the presence of RGD- or RGE-containing peptides or anti-human FN purified Igs. After 8 h, nonadherent cells were removed, and adherent cells were fixed and stained by crystal violet. Data are reported as the mean ± SD of absorbance at 540 nm from quadruplicate experiments.

whereas lower bTSH concentrations were ineffective. FN expression in serum-free cultures as well as CoGI expression with or without FCS were unmodified by bTSH treatment. Our results indicate that normal human thyroid cells produce ECM selectively when stimulated by serum. Although CoGI was not produced, FN was produced and deposited only upon serum stimulation.

Adhesion of thyroid cells in culture is mediated by integrins

We previously showed that normal human thyroid cells in culture express the integrin $\alpha_3\beta_1$, a FN receptor, and that this integrin is able to bind immobilized FN (16, 17). To demonstrate the relevance of the FN/integrin interaction in monolayer cultures of thyroid cells, we tested the ability of RGDSP peptides, specific inhibitors of integrin binding to FN, and anti-FN antibodies to inhibit cell adhesion (Fig. 4). Cells from 7-day-old cultures were cultured in 10% FCS containing medium in the presence of RGDSP peptide or RGESP peptide (control peptide unable to inhibit binding to FN) or anti-FN antibody. After 8 h, nonadherent cells were removed, adherent cells were observed by inverted phase contrast microscopy, and their number was determined by the crystal violet colorimetric method. Only scattered round cells were present in the wells containing RGD peptides, whereas in the control wells, cells had flat polygonal shape and large spreading. RGDSP peptide determined up to 90% inhibition of cell adhesion, and inhibition by anti-FN antibody reached 80%, whereas RGESP peptides had no blocking effect, demonstrating that FN is the major ECM component involved in thyroid cell anchorage in culture.

Adhesion to matrix is required for cell survival

The induction of apoptosis by denied adhesion to matrix and by serum starvation was investigated. Cells were plated in medium with 10% FCS in a plates coated with a thin layer of 2% agarose to prevent matrix deposition. After 24–96 h of culture, both floating and adherent cells were collected. DNA fragmentation was observed by DNA electrophoresis (Fig. 5), and loss of plasma membrane asymmetry before loss of membrane integrity was demonstrated by contemporary staining of the cells with annexin V and propidium iodide (Fig. 6), thus confirming apoptotic cell death. Hypodiploid cells were observed in the agar-coated plates by flow cytometry, and analysis by this method showed a time-dependent apoptosis (Fig. 7).

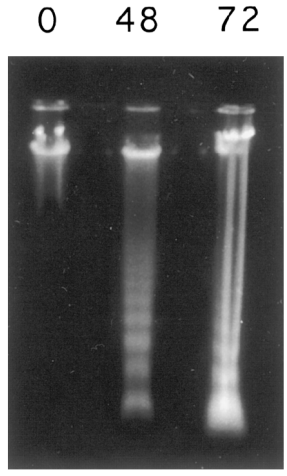

FIG. 5. Gel electrophoresis analysis of low molecular weight DNA from thyroid cells cultured for 0, 48, and 72 h in suspension in agar-coated plates.

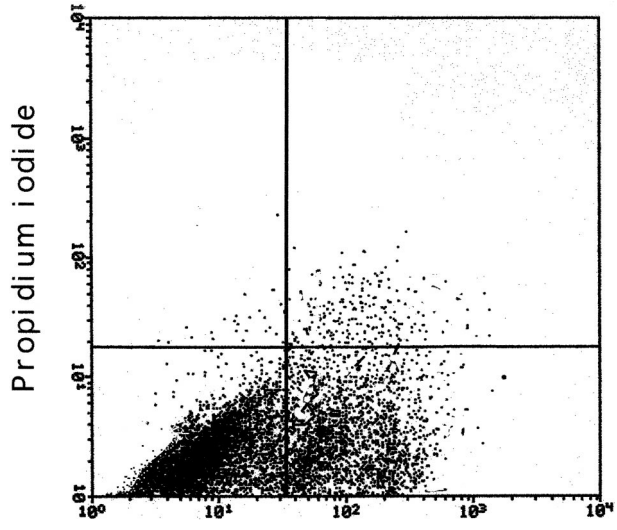

FIG. 6. Annexin V assay for determination of the apoptosis/necrosis ratio was performed by incubating the cells cultured for 48 h in suspension with annexin V-fluorescein conjugated (*abscissa*) and propidium iodide (*ordinate*) and analyzing them by flow cytometry. In the *lower left quadrant* are located intact cells; in the *lower right quadrant* are the apoptotic cells stained by annexin V and unstained by propidium iodide.

FCS is required to maintain insoluble FN deposits and cell survival

Degradation of ECM is known to be a critical event in organ regression induced by growth factor starvation (18). To determine whether degradation of FN occurs when serum is withdrawn, deposited FN was measured during serum starvation. Thyroid cells were plated in titration plates, untreated or coated with FN (5 μg/mL overnight), in F-12 medium in the presence of 10% FCS for 24 h. Then, medium was removed, and plates were washed and filled with fresh serum-free medium with or without 10 mU/mL bTSH. After 2, 4, 6, and 8 days of culture, the wells were fixed, and insoluble FN was measured by enzyme-linked immunoassay. After serum withdrawal, in uncoated wells in the absence of bTSH, the FN decreased progressively (Fig. 8), and

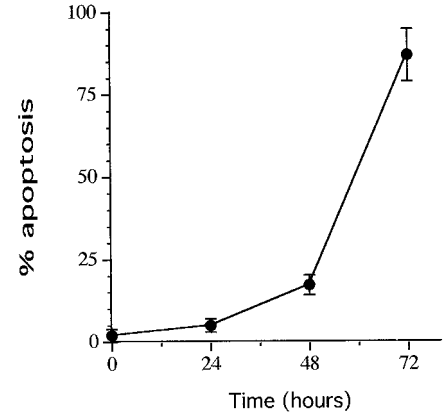

FIG. 7. Estimation of apoptosis by flow cytometry in thyroid cells cultured in suspension. Cells were plated in the presence of 10% FCS in agarose-coated plates. At various time intervals, adherent and floating cells were harvested, and apoptosis was determined by flow cytometry. A time-dependent increase in apoptotic hypodiploid cells was observed.

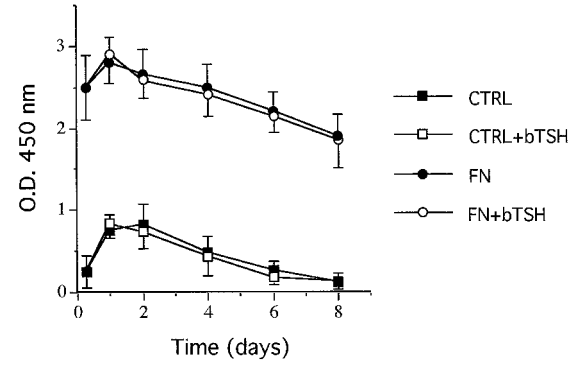

FIG. 8. FCS is required to maintain FN deposits. Normal thyroid cells were plated for 24 h in the presence of 10% FCS in plates untreated (CTRL) or coated with FN. Then FCS was removed, and cells were cultured in serum-free medium with or without bTSH. At the indicated time, the amount of insoluble FN was determined by enzyme-linked immunoassay.

cell shape changed from flat polygonal to spindle (not shown). After 8 days, FN reached the initial level, about 60% of the cells still adherent were completely round, and many were floating in the medium. In FN-coated plates, serum starvation did not affect cell shape, the cells remained flat, polygonal, and adherent; the decrease in FN was minimal, and by 8 days it was still present at high level. FN degradation was not affected by the presence of bTSH in the culture medium. A serum factor(s) trapped in the matrix or between the cells and the plate could account for the modest increase in FN production observed in all culture conditions after serum withdrawal.

In parallel experiments, cells were plated in serum-free medium in FN-coated or uncoated plates, both floating and adherent cells were collected at different times, and the presence of apoptosis was determined. DNA electrophoresis of cells cultured for 6 days in uncoated plates showed the characteristic fragmentation pattern observed in apoptotic cell death (not shown), and flow cytometric analysis demonstrated the presence of hypodiploid cells (Fig. 9A). Analysis of floating and adherent cells by flow cytometry showed the

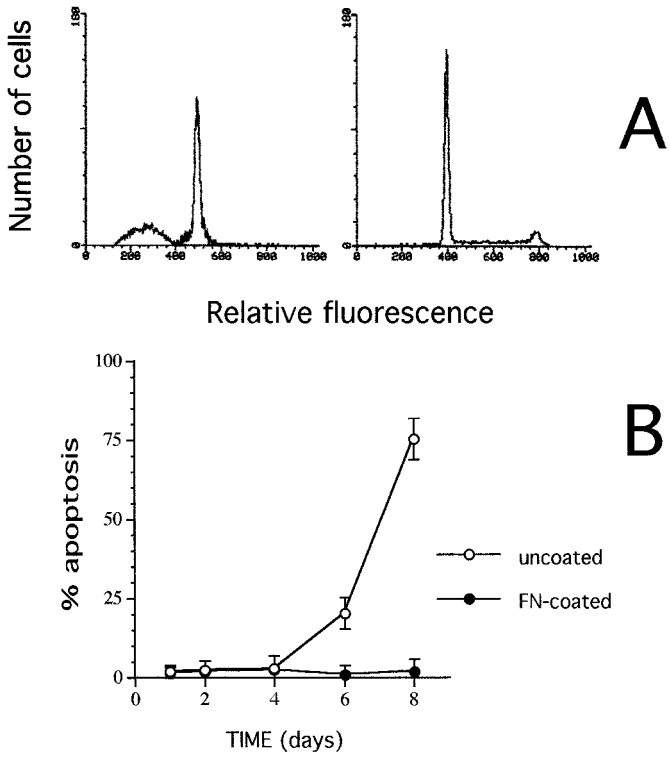

FIG. 9. A, Flow cytometric analysis of thyroid cells cultured on plates untreated (*left*) or coated with FN (*right*). After 6 days, floating and adherent cells were collected, stained with propidium iodide, and analyzed for DNA content by flow cytometry. B, Apoptotic cells with hypodiploid DNA content were gated, counted, and reported in the diagram as a percentage of apoptotic cells.

presence of a time-dependent increasing number of hypodiploid cells in uncoated plates, whereas they were absent in cultures from FN-coated plates (Fig. 9B).

Discussion

ECM regulates cell behavior and plays an important role in the regulation of many biological processes, including gene expression, differentiation, cell survival, and growth. Most normal cells undergo apoptosis when attachment to ECM is denied, whereas the anchorage dependence is changed in transformed cells. This type of programmed cell death, called anoikis (19–21), has been described in normal epithelial and endothelial cells and in transformed fibroblasts, whereas oncogenic transformation renders epithelial cells resistant (9, 22). Anoikis has also been described in a human fetal thyroid cell line immortalized *in vitro* by simian virus 40 infection (14). Although in a previous study we demonstrated that FN and CoGI stimulate the proliferation of normal human thyroid cells in primary culture, a direct demonstration of the role of ECM in the survival of primary thyroid cells in cultures was not addressed. The demonstration that thyroid cells require ECM to survive suggests a new fundamental role of the stromal component of the thyroid by which tissue structure is stabilized. Indeed, almost all thyrocytes are organized to form follicles surrounded by basement membrane, whose ECM and cellular basal pole tightly interact. A limited access to the matrix would determine a growth/death equilibrium, resulting in a constant number of cells. Under this model, thyroid hyperplasia generated by stimulatory factors, as in iodide-deficient intake (increased TSH) or Graves' disease (anti-TSH antibodies), must associate with stromal increase, whereas only oncogenic transformation can determine hyperplasia with poorly represented stroma. This statement is supported by the actual finding that hypercellularity with absent or poor stroma at histology is strongly suggestive of aggressive transformation.

Primary cultures of porcine thyroid cells and differentiated rat thyroid epithelial cells (FRTL-5) synthesize some ECM components, such as FN, type IV collagen, and laminin (23–25). In a previous study we demonstrated that the fetal thyroid cell line TAD-2 stimulated by serum deposits FN as insoluble matrix, and that FN is required for cytoskeletal organization and to prevent anoikis through a mechanism mediated by integrins (14). These results are now confirmed in primary thyroid cultures, confirming that the production and deposition of FN and its need for survival are general features of epithelial thyroid cells, not only of a fetal thyroid cell line.

Our results indicate that FN is selectively produced and deposited as insoluble matrix by normal human thyroid cells. Although CoGI was not produced, FN was produced and deposited only upon serum stimulation. FCS is a complex mixture of growth factors and other factors required by several cell types to survive in culture. Stimulation of fibroblasts by serum induces the transcription of several cytoskeleton and extracellular matrix genes, such as β-actin, α-tropomyosin, and FN (26, 27). Also, individual growth factors present in the serum, such as epidermal growth factor, insulin, platelet-derived growth factor, and transforming growth factor-β, strongly stimulate FN synthesis; thus, one or more of these factors, alone or in combination, could be responsible for serum-induced FN synthesis in thyroid cells (28). An alternative way in which the cells can regulate their interaction with ECM is remodeling their microenvironment by degrading the matrix. ECM-degrading proteinases correlate with metastasis and tumor growth by facilitating invasion of the cells through the matrix and are also involved in regulation of the development and function of normal tissues (29–31).

Proteolysis of basement membrane is responsible for involution of the mammary gland and provides direct evidence for the role of ECM in regulating functional differentiation of a hormone-dependent tissue (20). Loss of mammary gland function during involution is due to increased expression of metalloproteinases, stromelysin, and tissue plasminogen activator and can be regulated by their inhibitors (tissue inhibitor metalloproteinases and plasminogen activator inhibitor-1) (20, 32). More recently, urokinase-type plasminogen activator receptor has been shown in rat thyroid cells as well as in human TAD-2 and transformed thyroid cells, thus becoming a candidate for ECM remodeling in the thyroid (33–35).

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