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Fibronectin Is Required to Prevent Thyroid Cell Apoptosis through an Integrin-Mediated Adhesion Mechanism*

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ABSTRACT

Apoptosis or programmed cell death occurs in a wide variety of cell types when adhesion to extracellular matrix (ECM) is denied. Invasion and metastasis by tumor cells involve the loss of normal cell-ECM contacts and require independence from such control mechanisms. We studied whether the immortalized thyroid cell line TAD-2 is a model suitable to investigate thyroid cell-ECM interaction, and we analyzed the role of integrin-fibronectin (FN) interaction in apoptosis. Adhesion, spreading, and cytoskeleton organization in TAD-2 cultured cells were dependent upon integrin-FN interaction. Cell spreading and cytoskeletal organization were coupled to deposition of insoluble FN induced by serum. Expression of integrin-FN receptors was demonstrated by flow cytofluorometry with specific antibodies, and strong integrin-dependent adhesion was demonstrated by attachment assays to immobilized FN. Apoptosis, occurring in different culture conditions, was determined by cell morphology and DNA electrophoretic analysis and quantitated by flow cytometry in propidium iodide-stained cells. Thyroid cells underwent apoptosis in the presence of serum when adhesion was prevented by specific peptides that inhibit integrin binding to FN (RGD-containing peptides) or by coating the culture plates with agar. In serum-free cultures, apoptosis was prevented by insoluble FN immobilized on the plates, but not by soluble FN. These results suggest that the TAD-2 cell line is a good model to study thyroid cell-ECM interaction, that FN, assembled into insoluble matrix, is required for cytoskeletal organization and to prevent thyroid cell apoptosis, and that integrin-mediated adhesion is involved in this process. (*J Clin Endocrinol Metab* **83:** 3673–3680, 1998)

THYROID tumors comprise benign nonprogressive adenomas, well differentiated papillary and follicular carcinomas, and the highly invasive anaplastic carcinoma. Although a sequence of gene mutations responsible for thyroid cell transformation has been identified, it is not clear why some neoplastic phenotypes are more invasive than others. Invasion and metastasis by tumor cells involve the loss of normal contacts with the extracellular matrix (ECM) surrounding the thyroid follicles. Most normal cells undergo apoptosis when attachment to ECM is denied (anoikis) (see Ref. 1 for review), whereas the anchorage dependence is reduced or totally absent in transformed cells. The response to denied ECM attachment appears to be cell type specific. While nontransformed primary fibroblasts in suspension undergo cell cycle arrest, Myc/Ras and E1A/Ras transformation sensitizes to apoptosis, and Src transformation renders fibroblasts resistant (2). On the contrary, normal epithelial cells appear to be triggered into apoptosis as a result of detachment, whereas their oncogenic transformation seems to have a protective effect. We studied whether the immortalized thyroid cell line TAD-2 is a model suitable to investigate thyroid cell-ECM interaction, and we analyzed the role of integrin-fibronectin (FN) interaction in apoptosis.

Apoptosis or programmed cell death is an active process of self-destruction that requires the activation of a genetic program leading to changes in morphology, DNA fragmentation by an endogenous deoxyribonuclease, and protein cross-linking (3, 4). Although the molecular mechanisms behind apoptosis remain poorly understood, some molecular effectors have been identified. It is now clear that the apoptotic pathway can initiate at the cell surface from membrane receptors such as Fas/APO1 and tumor necrosis factor receptor-1, and that cysteine proteases represent one of the effector components of the apoptotic machinery. Activation of the apoptotic pathway is under the control of physiological stimuli such as environmental signals, cytokines (5–7), and growth factors and can also be induced by pathological stimuli, radiation, and anticancer drugs (2, 8, 9). Hormone depletion determines apoptosis in a number of hormone-dependent tissues, such as prostate and mammary glands (10, 11) or uterine epithelium. Also, serum withdrawal in endothelial cells as well as in canine thyroid primary cultures and Kirsten-*ras*-transformed rat thyroid cells induces programmed cell death (12, 13). Like hormones and growth factors, the ECM affects cell behavior and plays an important role in the regulation of many biological processes, including cell morphology, differentiation, transformation, and growth (14, 15). Recent studies demonstrate that in addition to reg-

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ulating cell growth and differentiation, ECM is also a survival factor for many cell types. Most normal cells require attachment to ECM to survive, whereas the anchorage dependence is reduced or totally absent in transformed cells. Cell adhesion to ECM is mainly mediated by the integrins, a family of cell surface receptors widely expressed on all tissues. The $\alpha\beta$ integrin complex has an extracellular domain bearing the ligand-binding site and an intracellular domain interacting with cytoskeletal proteins (16). Some of these receptors colocalize with several regulatory proteins, such as $pp60^{src}$, $pp125^{FAK}$, protein kinase C, and mitogen-activated protein kinase, in a subcellular structure defined as focal adhesion (17). From these subcellular sites initiates the signal transduction pathway triggered by the integrin-ECM interaction that contributes to the regulation of many biological processes, including differentiation, transformation, and growth (14, 18). We previously showed that integrin activation by ECM regulates cytoskeletal organization and stimulates the proliferation of normal human thyroid cells in culture (19). In the present study we demonstrate that the immortalized thyroid cell line TAD-2 is a good model to study thyroid cell-ECM interaction. TAD-2 cells stimulated by serum deposit FN as insoluble matrix required for cytoskeletal organization and to prevent apoptosis through integrin-mediated adhesion.

Materials and Methods

Cell culture

The TAD-2 cell line, obtained by simian virus 40 infection of human fetal thyroid cells (20), was donated by Dr. T. F. Davies, Mount Sinai Hospital (New York, NY), and cultured in a 5% CO_2 atmosphere at 37 C in RPMI medium supplemented with 10% FCS. Medium was changed every 3–4 days. Cells were detached by 0.5 mmol/L ethylenediamine tetraacetate in calcium- and magnesium-free phosphate-buffered saline (PBS) with 0.05% trypsin.

Antibodies, immunofluorescent localization, and flow cytometric analysis

For intracellular immunofluorescence (cytokeratin and thyroglobulin), cells were fixed in 3.5% paraformaldehyde, 0.2% Tween-20 in PBS, washed twice in PBS, and resuspended in 0.5% BSA-PBS; immunostaining was then performed using fluorescein-conjugated anticytokeratin antibodies (Ortho, Raritan, NJ) or rabbit antihuman thyroglobulin serum followed by sheep antirabbit IgG as a fluorescein-conjugated secondary antibody. Serum from nonimmunized rabbits or nonspecific fluoresceinated Igs of the same isotype were used as controls. Cells were then analyzed by flow cytometry using a FACScan apparatus (Becton Dickinson Co., Mountain View, CA).

Monoclonal antibodies of mouse origin against the various integrin subunits were donated as indicated: A1A5 (anti-β_1), Dr. M. E. Hemler (Boston, MA); J143 (anti-α_3), Dr. L. J. Old (New York, NY); and E7P6 (anti-β_6), Dr. D. Sheppard (San Francisco, CA). Monoclonal antibodies to α_5 and α_v were purchased from Telios (San Diego, CA); anti-$\alpha_v\beta_3$ and anti-$\alpha_v\beta_5$ were purchased from Chemicon (Temencula, CA); fluorescein-conjugated antimouse and antirabbit IgG and horseradish peroxidase-conjugated antirabbit IgG were purchased from Ortho (Raritan, NJ). Rabbit polyclonal antibodies to human FN, collagen (CoG), vitronectin (VN), and laminin (LM) were purchased from Chemicon.

Cells were plated onto sterile glass coverslips and cultured for up to 72 h at 37 C in RPMI-10% FCS. Cells were rinsed in PBS, fixed in 3.5% paraformaldehyde in PBS for 10 min, incubated in 0.5% Triton X-100 for 10 min, and blocked in 0.5% BSA for 10 min. Cells were incubated with phycoerythrin-conjugated phalloidin (Sigma) or primary antibody in PBS-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-50% glycerol, and observed with a fluorescence microscope (Zeiss, Oberkochen, Germany). Flow cytometric analysis was performed as follows. Cells harvested from subconfluent cell cultures by trypsin-PBS were incubated with the primary monoclonal antibody for 1 h at 4 C in 0.5% BSA-PBS, washed in the same buffer, and incubated again with the secondary fluorescein-conjugated antibody for 30 min at 4 C. Cells were resuspended in BSA-PBS and analyzed by flow cytometry. Nonspecific Igs of the same isotype were used as controls. The expression of each integrin was represented as the relative fluorescence index (RFI) = experimental mean fluorescence/control mean fluorescence.

Enzyme-linked immunoassay

Cells were plated in 96-well flat-bottomed microtiter plates (Costar, Cambridge, MA) in RPMI with or without serum, and after an appropriate time, the 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 room temperature. Then, the plates were washed with PBS, filled with 100 μL horseradish peroxidase-conjugated antirabbit IgG in PBS and 0.2% Tween-20, allowed to react for 1 h, washed again 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.

Cell attachment assay

The assay was performed in 96-well flat-bottomed microtiter plates. The wells were filled with 100 μL of the appropriate dilution in PBS of FN (Collaborative Research, Bedford, MA). After overnight incubation at 4 C, the plates were washed with PBS, filled with 100 μL 1% heat-denatured BSA, and incubated for 1 h at room temperature. Then, plates were washed and filled with 100 μL/well PBS, 0.9 mmol/L $CaCl_2$, and 0.5 mmol/L $MgCl_2$ containing 7×10^4 cells. After 30 min at 37 C, plates were gently washed three times 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.

In the adhesion inhibition assay, 5×10^4 cells/well were coincubated with 100 or 500 μg/mL 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) in plates previously coated with 2 μg/mL FN. All 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 (TTE) for 20 min at 4 C; and centrifuged at 13,000 rpm for 30 min. Centrifugation-resistant low mol wt DNA was extracted with phenol-chloroform, precipitated with ethanol, and incubated with 0.5 μg/mL deoxyribonuclease-free ribonuclease for 30 min at 37 C. DNA with loading buffer was electrophoresed in 1% agarose and 1 μg/mL bromide at 50 V in 45 mmol/L Tris-borate and visualized by UV.

Cytofluorometric estimation of apoptosis was performed as described previously (21). 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 deoxyribonuclease-free 10 μg/mL ribonuclease A overnight at 4 C. Cells were then analyzed by flow cytometry using a FACScan (Becton Dickinson Co.).

Statistics

Results are presented as the mean ± SD. SDs less than 10% are not reported in the diagrams.

Results

TAD-2 cell line characterization

The thyroid epithelial nature of the TAD-2 cell line was confirmed by flow fluorocytometry using anticytokeratin and antithyroglobulin antibodies. The presence of cytokeratin in the cells ascertained the epithelial origin of the TAD-2 cell line (Fig. 1A). Antithyroglobulin antibodies weakly stained the cells, demonstrating, as expected, a low thyroglobulin content (Fig. 1B), as TAD-2 cells originate from fetal thyroid, and TSH was not present in the culture medium.

Anchorage of thyroid cells in culture is mediated by FN-integrin interaction

The cell monolayer in *in vitro* culture is determined by cell interaction with insoluble ECM components recruited from the serum and/or produced by the cells themselves. To determine whether thyroid cell anchorage was an integrin-RGD-dependent mechanism, 5×10^4 TAD-2 cells/well, obtained from subconfluent cultures by mild trypsin treatment, were plated in 96-well flat-bottomed microtiter plates in 100 μL RPMI-1% FCS. The cells were cultured in the presence of synthetic peptides containing the sequence RGDSP that inhibit integrin binding to both FN and VN, or in the presence of control peptides containing the sequence RGESP or anti-ECM-purified Igs (Fig. 2). After 8 h, nonadherent cells were removed by gentle washing, adherent cells were observed by inverted phase contrast microscope, and their number was determined as described in *Materials and Methods* for the cell attachment assays. Only a few round cells were present in the wells containing RGDSP peptides or anti-FN antibody, whereas in the presence of RGESP-containing peptides, the majority of the cells were adherent and acquired a flattened shape. Cell adhesion was inhibited by RGDSP peptides (92% inhibition), whereas RGESP peptides had no blocking effect, demonstrating that cell anchorage to the plate was mediated by a RGD-dependent FN/VN-integrin interaction. The anchorage inhibition obtained by anti-FN antibodies was only slightly lower (88% inhibition), suggesting that FN is the major ECM component involved in TAD-2 cell anchorage, although a minor role for VN or other matrix proteins cannot be excluded.

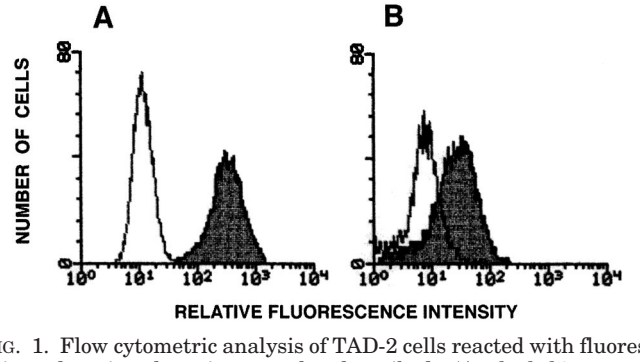

FIG. 1. Flow cytometric analysis of TAD-2 cells reacted with fluoresceinated anticytokeratin monoclonal antibody (A, *dark histogram*) and with rabbit antihuman thyroglobulin serum followed by fluorescein-conjugated sheep antirabbit IgG (B, *dark histogram*). The *light histograms* represent the negative controls (nonspecific fluoresceinated mouse Igs and serum from nonimmunized rabbits).

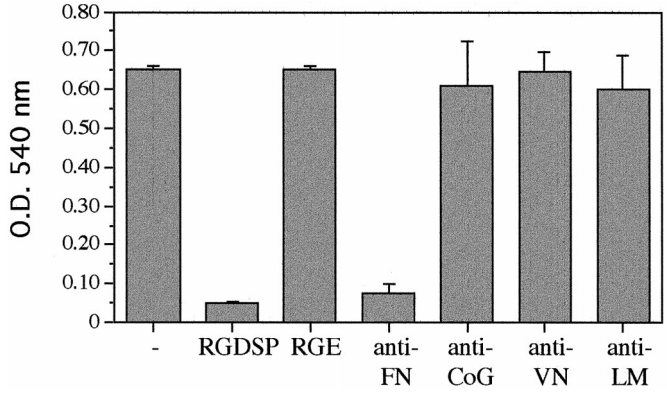

FIG. 2. Inhibition of thyroid cell adhesion in serum-containing cultures. A total of 3×10^4 TAD-2 cells/well were plated in flat-bottomed microtiter plates in RPMI-1% FCS in the presence of RGD- or RGE-containing peptides or antihuman FN-, CoG-, VN-, or LM-purified Igs. After 8 h, nonadherent cells were removed, and adherent ones were fixed and stained with crystal-violet. After destaining, the absorbance at 540 nm was measured. Cell adhesion was inhibited by the RGDSP peptide, a specific inhibitor of integrin binding to FN, and by anti-FN antibody, whereas the RGESP peptides and other anti-ECM antibodies had no blocking effect. Data are reported as the mean ± SD of quadruplicate experiments.

Cytoskeletal organization and insoluble FN deposition

Cells were plated in RPMI in the presence of 10% FCS on coverslips and cultured for up to 72 h (Fig. 3). As expected, increasing the time of culture resulted in a progressive flattening of cells and actin microfilament organization. Cortical actin organized first, followed by a progressively more complex cytoskeletal organization. A diffuse staining was observed with anti-FN antibody after trypsin treatment in uncultured cells (not shown). Trypsin treatment of cultures did not completely remove the FN present on the cell surface, and intracellular FN was also present in cultured cells 1 h after plating. Then, increasing the time of culture resulted in progressive organization and deposition of FN fibrils. After 72 h of culture, the cells were wrapped in a dense net of FN fibrils deposited on both lower and upper sides of the cells. The cells were also plated and cultured in medium containing 0.2% BSA in the absence of FCS (not shown). In serum-free cultures, the cells required several hours to became adherent and an even longer time to spread and organize actin microfilaments. Cells deposited FN fibrils, but their staining remained dim even after 3 days of culture.

Thyroid cells deposit insoluble FN in serum-containing cultures

A total of 3×10^4 cells were plated in triplicate wells in RPMI medium-0.2% BSA with or without 1 mg/mL soluble FN or with 1% FCS. At different times wells were washed, and adherent cells and insoluble deposited matrix were fixed. By enzyme-liked immunoassay with antihuman FN, the total amount of FN present in the cells or deposited onto the bottom of the wells was estimated (Fig. 4). After 1 h, FN was already detectable in the FCS-containing wells, and increasing the time of culture resulted in a progressive FN deposition. Serum is a rich FN solution, and a 1% FCS so-

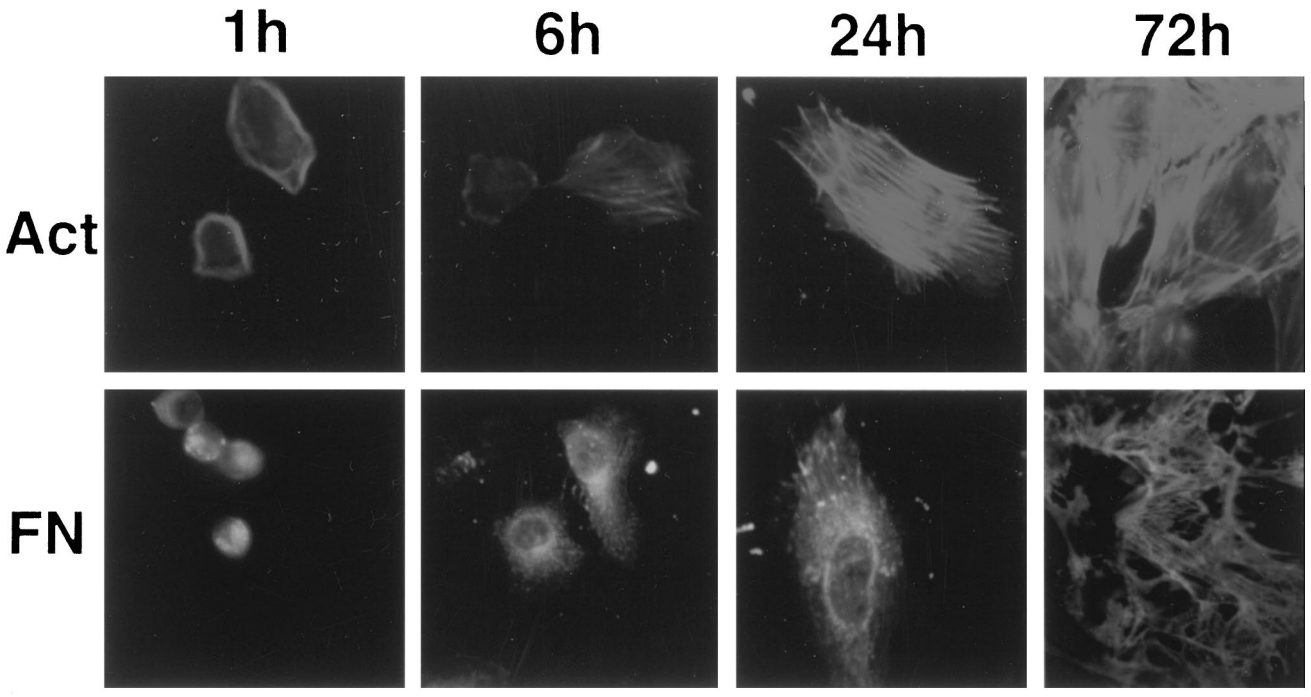

FIG. 3. Cytoskeletal organization, cell spreading, and deposition of insoluble FN. Thyroid cells were plated in 10% FCS-containing medium on coverslips and cultured for up to 72 h. Increasing time of culture resulted in a progressive flattening of cells and actin microfilament organization (Act) as well as in progressive organization and deposition of insoluble FN fibrils (FN).

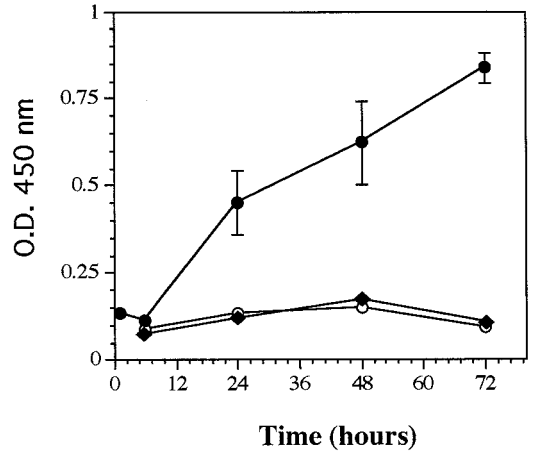

FIG. 4. Thyroid cells deposit FN. A total of 3×10^4 TAD-2 cells/well were plated in flat-bottomed microtiter plates in RPMI containing 1% FCS (*solid circles*), 0.2% BSA (*open circles*), or 0.2% BSA-10 μg/mL FN (*solid squares*). After an appropriate time, the wells were gently washed, fixed with methanol-acetone, and air-dried. The presence of FN was estimated by enzyme-linked immunoassay using a polyclonal antihuman FN. The relative FN content per well is expressed as the mean absorbance ± SD of triplicate wells. In the absence of FCS, the cells were not adherent until 6 h of culture; thus optical density determination at 1 h is not reported in the diagram.

lution contains about 3 μg/mL FN. In serum-free medium containing human FN, FN was not deposited as insoluble matrix, demonstrating that TAD-2 cells were not able to recruit FN from the medium. In the absence of FCS, cells were not adherent until 6 h and were removed by PBS washing. After that time, a low amount of FN that remained constant during the culture was detected.

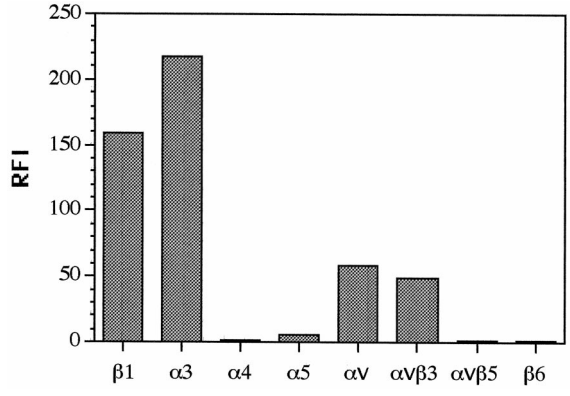

FIG. 5. Expression of integrin receptors in TAD-2 cells from subconfluent cultures. Cells were harvested by mild trypsinization from subconfluent cultures and reacted with monoclonal antibodies specific for single integrin subunits (β_1, β_6, α_3, α_4, α_5, and α_v) or whole receptors ($\alpha_v\beta_3$ and $\alpha_v\beta_5$) followed by the secondary fluorescein-conjugated antibody. The relative fluorescence values were measured by flow cytometry as described in *Materials and Methods*. The expression of each integrin heterodimer or single subunit is reported as RFI = experimental mean fluorescence/control mean fluorescence.

Expression of integrin-FN receptors and their localization

The expression of integrin-FN receptors was evaluated by flow cytometry with monoclonal antibodies specific for the β_1, β_6, α_3, α_4, α_5, α_v, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ chains. Figure 5 reports the average expression of integrin heterodimers and integrin subunits measured in TAD-2 cells cultured at subconfluence. α_3 was the most abundant α integrin subunit (RFI = 218 ± 16.2); α_v was strongly expressed, although at a lower level (RFI = 58.0 ± 2.1); whereas α_5 was only slightly expressed (RFI = 6.1 ± 0.9), and α_4 and β_6 were totally absent. Mono-

clonal antibodies to whole integrin heterodimers detected the presence of $\alpha_v\beta_3$, whereas the VN receptor $\alpha_v\beta_5$ was totally absent. Cells cultured for 3 and 72 h on coverslips in medium supplemented with FCS were fixed and stained by indirect immunofluorescence with antipaxillin, β_1, α_3, α_5, α_v, and $\alpha_v\beta_3$ antibodies (Fig. 6). Paxillin, α_v, and $\alpha_v\beta_3$ clearly localized in large focal contacts, whereas anti-β_1 and anti-α_3 antibodies produced a fine dotted staining fairly distributed on the entire cell surface. α_5 was also localized in focal contacts, but its staining was extremely dim compared with that of α_v or paxillin (not shown).

Adhesion to immobilized FN is mediated by integrins

Cell attachment assays were performed in 96-well flat-bottom microtiter plates coated with different concentrations of human FN (Fig. 7A). Thyroid cells from subconfluent cultures showed a concentration-dependent adhesion to FN, reaching a maximum at about 12.5 μg/mL. The substrate concentration required to achieve 50% of the maximal cell adhesion was about 2 μg/mL FN.

Integrin involvement in thyroid cell adhesion to immobilized FN was investigated by an RGD-mediated attachment inhibition assay (Fig. 7B). Cell adhesion to FN was completely blocked in the presence of 100 μg/mL RGDSP or 500 μg/mL RGDTP peptides, whereas RGESP peptides had no effect.

Inhibition of integrin-mediated adhesion induces apoptosis

A total of 5×10^5 TAD-2 cells were plated in medium with 10% FCS, 10% FCS in a plate coated with a thin layer of 2% agarose to prevent matrix deposition, 0.2% BSA without FCS in a FN-coated plate (10 μg/mL in PBS overnight), or 50 μg/mL soluble FN-containing medium without serum. After a few hours in the presence of FCS and in the FN-coated plates, cells were adherent and acquired a flattened shape, whereas in the agar-coated plates and in the presence of soluble FN, the cells remained nonadherent, solitary cells showed a spherical conformation, and the majority clustered to form large cell aggregates floating in the medium.

After 24–96 h of culture, both floating and adherent cells were collected, and apoptosis was estimated by flow cytometric analysis. Hypodiploid cells were observed in the agar-coated plates and in the presence of soluble FN, whereas they were not evident in the presence of FCS or in the FN-coated plates. DNA fragmentation observed by DNA electrophoresis confirmed apoptotic cell death (not shown). Apoptosis, estimated by flow cytometric analysis, showed a time-dependent increment that reached 95% after 72 h of culture in the agar-coated plates in the presence of FCS as well as of soluble FN (Fig. 8). Similar results were obtained after 48 h of culture in serum-containing medium when adhesion to FN was inhibited by RGD-containing peptides (Fig. 9). The cells were plated in RPMI medium and FCS with or without

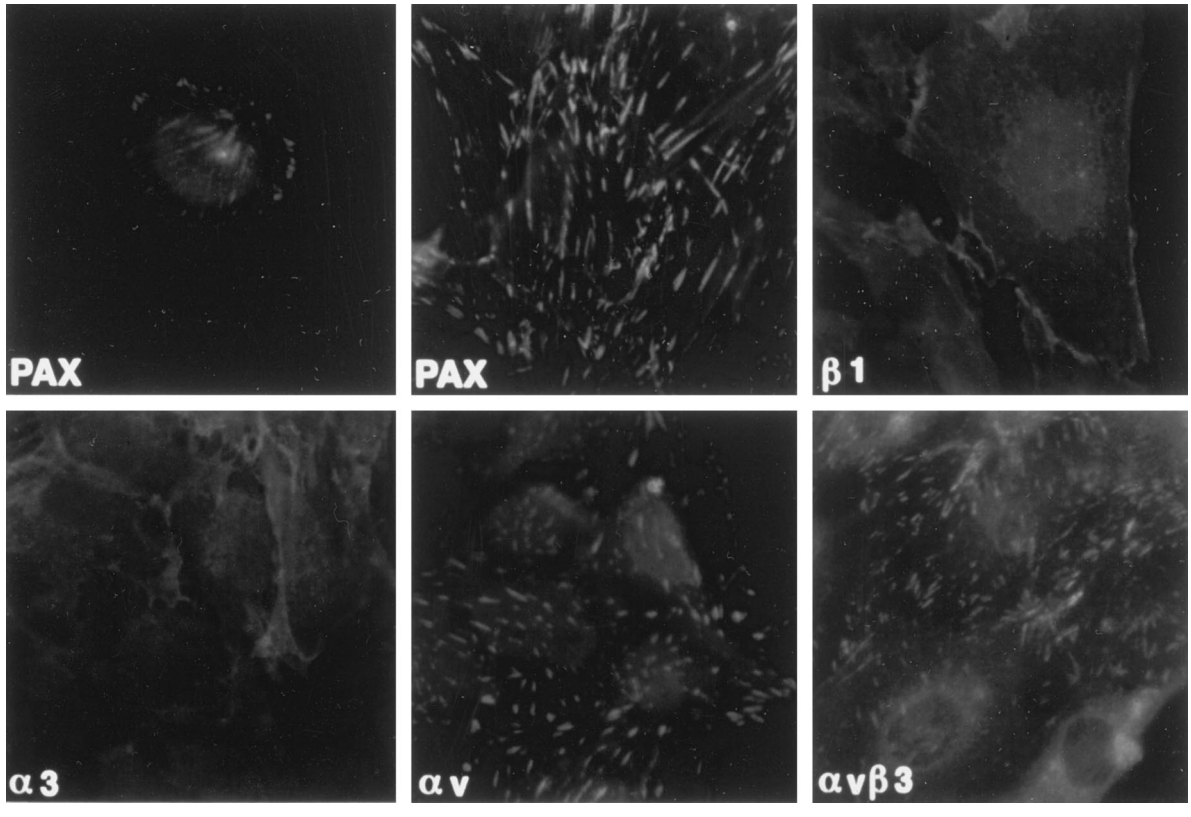

FIG. 6. Immunolocalization of integrin receptors in TAD-2 cells. The cells were cultured in 10% FCS-containing medium on coverslips for 3 h (*top left*) or 72 h and then fixed with paraformaldehyde, permeabilized by Triton X-100, and stained by indirect immunofluorescence with monoclonal antibodies. Localization of paxillin (Pax), α_v, and $\alpha_v\beta_3$ to large focal adhesions was clearly visible, whereas β_1 and α_3 failed to localize to focal contacts. *Top left*, Paxillin localization after 3 h of culture.

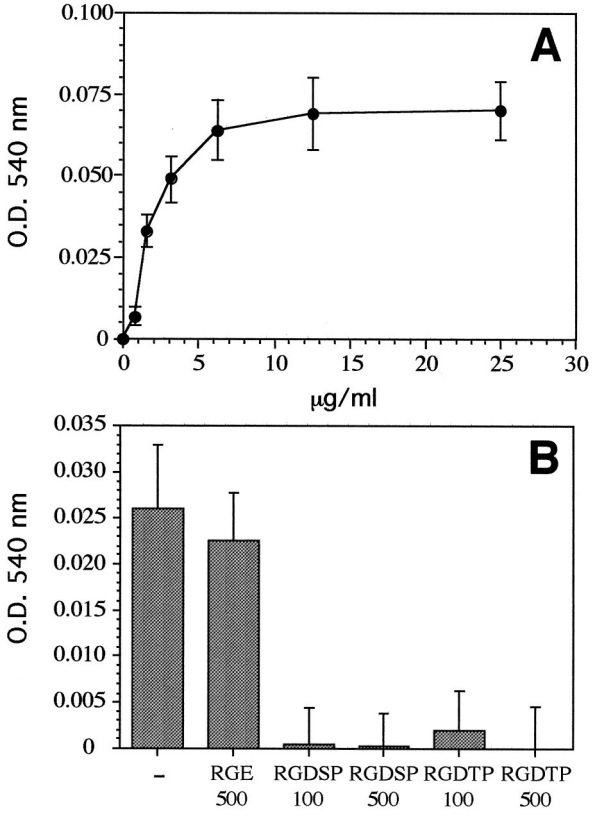

FIG. 7. Cell attachment to FN and its inhibition by synthetic peptides. A, Microtiter wells were coated with different concentrations of human FN and saturated with heat-denatured BSA. Cells in calcium- and magnesium-containing PBS were added and incubated at 37 C for 30 min. Attached cells were measured as described in *Materials and Methods*. B, Microtiter wells were coated with 2 μg/mL FN and saturated with BSA. Cells were added to the plates together with 100 or 500 μg/mL of RGD- or RGE-containing peptides. After 30 min at 37 C, the plates were gently washed, and attached cells were measured as described above. All experiments were performed in quadruplicate. Data are reported as the mean absorbance ± SD.

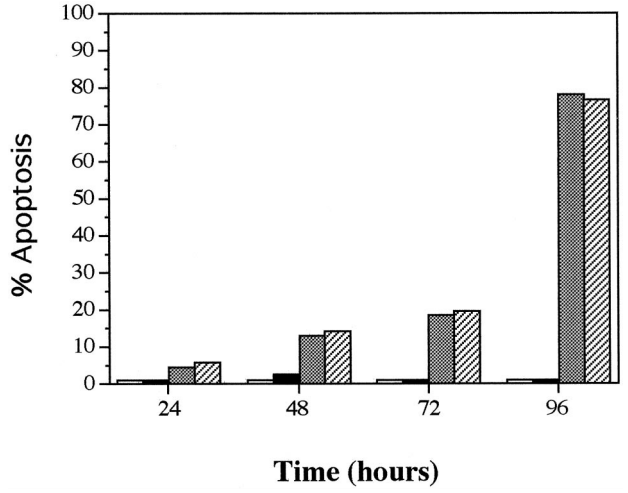

FIG. 8. Estimation of apoptosis by flow cytometry in TAD-2 cells cultured in 10% FCS in untreated plates (*open bars*), with 0.2% BSA, without serum, in FN-coated plates (solid bars), with 10% FCS in agar-coated plates (*shaded bars*), and with 50 μg/mL soluble FN and 0.2% BSA without serum (*slashed bars*). Culture plates were untreated or coated by overnight incubation with PBS and 10 μg/mL FN or on a thin layer of 2% agarose. At various time intervals, adherent and floating cells were harvested, and apoptosis was determined by flow cytometry. Time-dependent apoptosis was observed only in agar-coated plates in the presence of FCS and in soluble FN-containing medium.

100 μg RGDSP, RGDTP, or RGE peptides, and each 24 h, 100 μg more of the peptides were added to the culture. Alternatively, the cells were cultured in the absence of peptides in an agar-coated plate. In the presence of RGD peptides, as in the agar-coated plates, the cells remained nonadherent and acquired a spherical conformation or clustered to form floating aggregates. Under these experimental conditions, inhibition of cell adhesion induced apoptosis. RGE-containing peptides did not affect cell adhesion and did not induce apoptosis.

Discussion

Basement membrane is a complex structure composed of a number of extracellular matrix proteins that underlies epithelial and endothelial cells, thus providing a structural, fundamental support in maintaining orderly tissue organization. Follicular cells in the thyroid are surrounded by a continuous rim of matrix proteins whose major components are FN and LM (22). Although it is accepted that the thyroid basement membrane is produced by different cell types, including follicular cells, its metabolism is largely unknown.

ECM deposition and basement membrane formation occur in cultures of many cell types. The ability of primary cultures of porcine thyroid cells to synthesize FN and type IV collagen has been described (23, 24). The differentiated rat thyroid epithelial cell line FRTL-5 synthesizes, secretes, and organizes an ECM containing FN, CoG, and also LM (25). Loss of or change in the synthesis and assembly of matrix proteins follows cell transformation, and aberrant distribution of ectopic matrix is a frequent finding in tumors, including thyroid tumors (22).

As shown in Fig. 1, adherence of TAD-2 cells in culture is mediated by FN through one or more integrin receptors, although a minor role of other matrix proteins cannot be excluded. TAD-2 cells, like primary cultures of thyroid cells, can be cultured as a monolayer in the presence of serum. Although serum contains a large amount of FN that could be recruited by the cells, soluble FN was not used by TAD-2 to adhere, as demonstrated in the experiment with soluble human FN. Nevertheless, serum was required for active FN deposition. Trypsin treatment of cultures did not completely remove the FN present on the cell surface, as it could be detected by immunostaining after trypsin treatment in uncultured cells, but although the total amount of FN was very low and remained constant in serum-free cultures, it increased and was actively deposited as insoluble matrix in the presence of serum. This phenomenon was not restricted to thyroid cells and confirms previous observations in mesenchymal cells. Serum stimulation of quiescent fibroblasts induces coordinate transcriptional activity of several cytoskeleton and extracellular matrix genes. β-Actin, α-tropomyosin, and FN together with c-*fos* and c-*myc* belong to the class of the so-called early growth response genes whose expression is growth factor regulated (26, 27). Also in AKR-2B cells, FCS,

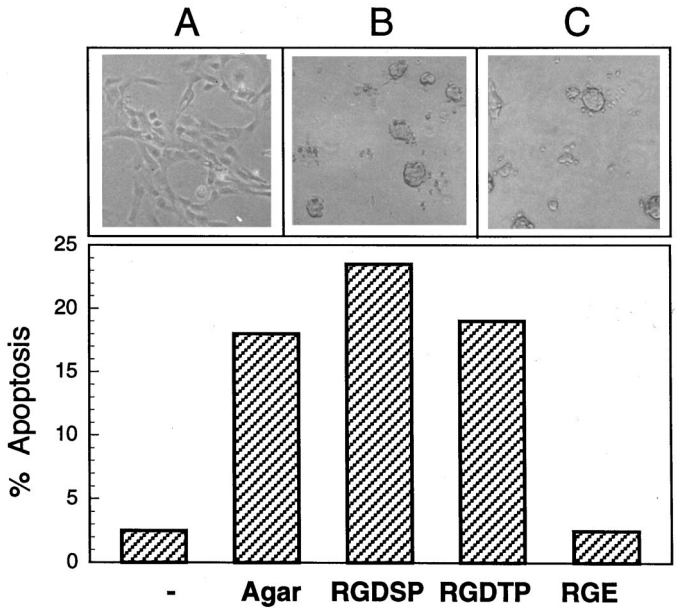

FIG. 9. Induction of apoptosis by RGD-containing peptides. Cells were plated in medium, with 5% FCS alone (−), or with RGDSP, RGDTP, or RGE peptides to inhibit integrin-mediated cell adhesion to FN or in the absence of peptides in a agar-coated plate (Agar). After 48 h of culture, adhesion and spreading in the presence of FCS (A) were inhibited in the agar-coated plates (B) as well as in the presence of RGDSP (C) and RGDTP (not shown) peptides, and cells acquired a spherical conformation or clustered to form nonadherent aggregates. Adherent and floating cells were collected, and apoptosis was estimated by flow cytometry (*bar graph*). The inhibition of adherence induced by agar coating and by blocking peptides induced cell apoptosis.

epidermal growth factor, insulin, platelet-derived growth factor, transforming growth factor-β, and phorbol esters are mitogenic and strongly stimulate FN, c-*fos*, and c-*myc* gene transcription within few minutes, thus determining cell proliferation and increased synthesis of FN (28).

As previously described in thyroid cells in primary cultures obtained from thyroids of adult subjects (19), in TAD-2 cells, $\alpha_3\beta_1$ integrin was the most abundant FN receptor. This integrin was distributed on the entire cell surface, but did not localize in the focal contacts. This surface-diffused distribution is characteristic of the $\alpha_3\beta_1$ integrin, and it has been described in a number of cell types, such as fibroblasts and epithelial cells (29). Although several studies have demonstrated a role of different FN receptors in regulating cell proliferation and cell survival, none has demonstrated such functions of the $\alpha_3\beta_1$ integrin; thus, this receptor has to be considered to play only a structural role, whereas its signaling capacity is not yet established (18, 30–34).

Although the α_5 chain was only minimally expressed, α_v was strongly represented. The α_v integrin subunit associates with several β-subunits, including β_1 (35), β_3 (36), β_5 (37), β_6 (38), and β_8 (39), generating monospecific receptors that bind only to FN or VN ($\alpha_v\beta_1$ and $\alpha_v\beta_5$, respectively) and receptors with multiple specificity interacting with both substrates ($\alpha_v\beta_3$ and $\alpha_v\beta_6$). The $\alpha_v\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_6$ receptors colocalize with paxillin, $p125^{FAK}$, and other molecules into adhesion plaques, transmembrane structures linking the ECM with components of the cytoskeleton (40). Although antibodies specific to $\alpha_v\beta_1$ were not used in our study, the lack of immunolocalization of the β_1-subunit into the focal contacts suggests that the expression of $\alpha_v\beta_1$ was very poor or totally absent in TAD-2 cells. Among the other α_v-subunit-containing integrins, only $\alpha_v\beta_3$ was expressed, whereas $\alpha_v\beta_6$, a FN receptor expressed predominantly by epithelial cells (41) and $\alpha_v\beta_5$ (that binds VN) were not found. The matrix interactions with $\alpha_5\beta_1$ and $\alpha_v\beta_3$ promote cellular responses such as proliferation and cell spreading (36, 42, 43). In the present study we did not determine the specific contribution of each integrin receptor; however, the experiments with RGD-containing peptides supported the evidence that FN exerts its biological role through integrins, because all these receptors interact with their ligands in an RGD-dependent manner (34, 44). After binding to their ligands, integrins cluster and promote the assembly of cytoskeleton, actin microfilament polymerization, and cell spreading. Endothelial and epithelial cells that are prevented from attaching to an ECM substrate do not organize their cytoskeleton and undergo apoptosis (45, 46). A number of studies report that proper cytoskeletal organization and cell shape, and not the simple occupancy of integrin receptors, are required to prevent apoptosis (45). Other studies demonstrated that apoptosis could be prevented by the binding of different types of integrins ($\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_2\beta_1$) to different substrates (FN, VN, and CoG), thus suggesting that this phenomenon is not restricted to a specific integrin but, rather, is generated by the ensuing cytoskeleton organization (47). Cell shape and cytoskeletal organization are required for anchorage-dependent cell survival, but this might not be sufficient, and specific intracellular signaling could be required. Apoptosis of CHO cells is not prevented by $\alpha_v\beta_1$ binding to FN, whereas $\alpha_5\beta_1$ binding to FN is effective in this regard, thus demonstrating the existence of specific signaling (48). This effect is independent of the level of $p125^{FAK}$ phosphorylation, whereas it is associated with increased Bcl-2 protein expression. The question of which integrin triggers signals that prevent thyroid cells from entering into a suicide program has not been addressed in the present study. As thyroid tumors display a changed profile of integrin expression (49), the elucidation of this question will be a valuable step toward a further understanding of the nature of thyroid tumor malignancy.

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