In vivo, thyroid follicles, the functional unit of the thyroid gland, are surrounded by mesenchymal tissue containing endothelial cells and fibroblasts. The extracellular matrix (ECM) in contact with epithelial cells exerts a profound influence upon function and behaviour of follicle cells. In vitro studies showed that ECM components play a role on adhesion, polarization, migration and confluence by a mechanism that did not involve cell proliferation. TGF-β1 strongly activated the production of thrombospondin-1 and αvβ3 integrin in a concentration-dependent manner whereas the expression of thyroglobulin was unaffected. Anisomycin, an inhibitor of protein synthesis, inhibited the effect of TGF-β1 on cell organization. Thrombospondin-1 reproduced the effect of TGF-β1. In the presence of thrombospondin-1 cells did not organize in follicle-like structures but, in contrast, spread and reached confluency independently of cell proliferation. This effect is suppressed by an RGD-containing peptide. The adhesive properties of thrombospondin-1 for thyroid cells were shown to be mediated by both the amino-terminal heparin-binding domain and the RGD domain of thrombospondin-1. Adhesion was shown to involve αvβ3 integrin. The results show that TGF-β1 exerted an influence upon function and behaviour of follicle cells partly mediated by the synthesis of thrombospondin-1 and of its receptor αvβ3 integrin.

Key words: Thyroid, TGF-β, Thrombospondin, Cell morphology

INTRODUCTION

In vivo, thyroid follicles, the functional unit of the thyroid gland, are surrounded by mesenchymal tissue containing endothelial cells and fibroblasts. The extracellular matrix (ECM) in contact with epithelial cells exerts a profound influence upon function and behaviour of follicle cells. In vitro studies showed that ECM components play a role on adhesion, polarization, migration and functional expression of thyroid epithelial cells in primary culture (Nitsch and Wollman, 1980; Chambard et al., 1981, 1983; Toda and Sugihara, 1990; Toda et al., 1995; Sasaki et al., 1991; Espanet et al., 1992). For example, in experiments where they were in contact with conventional adhesive substratum (polystyrene treated for tissue culture), thyroid epithelial cells, in the absence of thyrotropin (TSH), organized into monolayer (with their apical pole oriented towards the culture medium) or, in the presence of TSH, into three-dimensional follicle-like structures. This morphological TSH effect was originally described by Fayet et al. (1971). The formation of these two types of structures, monolayer or follicles, also depends on the substratum and on the cellular environment. In this respect, it was previously reported that thyroid epithelial cells adopted a follicular organization in the absence of thyrotropin when embedded in ECM gel. In type I collagen or in reconstituted basement membrane gel derived from the Engelbreth-Holm-Swarm (EHS) tumor, cells reorganized into properly oriented follicles while reexpressing normal thyroid functions (Chambard et al., 1981; Sasaki et al., 1991; Toda et al., 1990; Espanet et al., 1992). In contrast, when cultured on the surface of collagen gel, thyroid epithelial cells formed a monolayer similar to that obtained on plastic surfaces with the apical pole, facing the culture medium.

Little is known about the mechanisms of thyroid cell rearrangement from follicle-like structures to monolayers and how cell organization influences the synthesis and deposition of extracellular matrix components. Thyroid cells in primary culture have been shown to synthesize fibronectin (Giraud et al., 1981) heparan sulfate proteoglycans (Giraud and Bouchilloux, 1983; Wadeleux et al., 1985; Shishiba et al., 1988), type IV collagen (Wadeleux et al., 1985; Garbi et al., 1988), merosin (André et al., 1994) and thrombospondin-1 (Prabakaran et al., 1993; Bellon et al., 1994). In contrast to basement membrane components, particularly to merosin, the...
synthesis of thrombospondin was shown to be dependent on thyrocyte organization in culture. Our previous studies using porcine thyroid cells demonstrated an increase in thrombospondin-1 (TSP-1), a multifunctional protein implicated in a number of biological processes (for review see Lawler, 1986; Majack and Bornstein, 1987), in proliferating cells and in monolayers. Thyroid epithelial cells organized into follicle-like structures on plastic surface in the presence of TSH expressed little or no TSP-1 depending on the follicle-like structures on plastic surface in the presence of TSH, leading to the formation of TSH expressed little or no TSP-1 depending on follicle-like structures on plastic surface in the presence of TSH, leading to the formation of a single layer and stimulated the synthesis of TSP-1 in a concentration-dependent manner. The effect of TGF-β1 on cell organization was partly reproduced by TSP-1, added to the culture medium or coated on the plastic substratum, which promoted adhesion and migration of thyroid epithelial cells. This effect was prevented by RGD-containing peptide. The interactions between TSP-1 and thyroid cells involved both the α,β3 integrin and heparan sulfate receptors.

**MATERIALS AND METHODS**

**Isolation and culture of porcine thyroid cells**

Fresh porcine thyroid glands were obtained from Soirev (Rethel, France). Thyroid cells were dissociated by a discontinuous trypsin-EGTA treatment according to the method of Feyet and Lissitzki (1970), modified by Mauchamp et al. (1979). Five ml of freshly isolated cell suspension (3x10⁶ cells/ml) in Eagle’s minimum essential medium (MEM) (pH 7.4), containing 10% (v/v) foetal calf serum (FCS), TSH 1 mU/ml, penicillin (200 U/ml) and streptomycin sulfate (0.05 mg/ml), were seeded onto polystyrene flasks treated for tissue culture (Nunc, Copenhagen, Denmark) and incubated at 37°C in a 95% air-5% CO₂, water saturated atmosphere. Under these conditions, cells organized into follicle-like structures adhering to the plastic-treated surface. In experiments performed with TGF-β1, TGF-β1 at concentrations indicated in the text was added to the medium at the beginning of the culture period or after two days for varying periods as indicated. Thyroid cells were observed by phase contrast microscopy (Olympus IMT-2). The homogeneity of the epithelial cell population and the absence of fibroblasts were checked as previously described (Roger and Dumont, 1984). Bovine TSH (2 i.u./mg) and TGF-β1 were obtained from Sigma Chemical Company (St Louis, MO, USA).

**Cell labeling, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and autoradiography of labeled proteins**

Metabolic labeling of porcine thyrocytes was performed as previously described (Bellon et al., 1994) by incubation of the cells in fresh MEM supplemented with 2% (v/v) FCS, 2-aminopropionitrile fumarate (50 μg/ml), ascorbic acid (50 μg/ml) and 10 μCi/ml of L-[³⁵S]methionine and -cysteine, 2 ng/ml TGF-β1 and various concentrations of anisomycin (an inhibitor of protein synthesis) (Sigma). Control cells were incubated in the absence of TGF-β1.

At the end of the labeling periods, the medium was recovered and protease inhibitors (5 mg/l benzamidine, 2.5 mg/l pepstatin, 1 mg/l leupeptin and 1 mg/l aprotinin) were added. The culture medium was first dialyzed at 4°C against running tap water for 48 hours, then against distilled water for another period of 24 hours and finally lyophilized. The material was analyzed by SDS-PAGE on polyacrylamide gels (4% to 10% (w/v) acrylamide gradient or 4.5% (w/v) acrylamide or 7.5% (w/v) acrylamide) in 0.025 M Tris/0.192 M glycine/0.1% (w/v) SDS, pH 8.3, according to the method of Laemmli (1970). Samples were analyzed under non reducing or reducing conditions with 1% (v/v) 2-mercaptoethanol. The MW-SDS-200 kit (Sigma) was used as molecular mass markers. After SDS-PAGE, the gel was soaked in Amplify (Amersham, Les Ulis, France) for 30 minutes, dried under vacuum and processed for fluorography using Hyperfilm-MP (Amersham) and a Kodak X-Omatic intensifying cassette C-2 at ~80°C. Thyroglobulin (Tg or THG) and TSP-1 were measured from the gel by cutting off their radioactivity. For this purpose, the excised bands were homogenized against distilled water for another period of 24 hours and finally lyophilized. The material was analyzed by SDS-PAGE on polyacrylamide gels (4% to 10% (w/v) acrylamide gradient or 4.5% (w/v) acrylamide or 7.5% (w/v) acrylamide) in 0.025 M Tris/0.192 M glycine/0.1% (w/v) SDS, pH 8.3, according to the method of Laemmli (1970). Samples were analyzed under non reducing or reducing conditions with 1% (v/v) 2-mercaptoethanol. The MW-SDS-200 kit (Sigma) was used as molecular mass markers. After SDS-PAGE, the gel was soaked in Amplify (Amersham, Les Ulis, France) for 30 minutes, dried under vacuum and processed for fluorography using Hyperfilm-MP (Amersham) and a Kodak X-Omatic intensifying cassette C-2 at ~80°C. Thyroglobulin (Tg or THG) and TSP-1 were measured from the gel by cutting off their corresponding Coomassie blue stained band and measuring the radioactivity. For this purpose, the excised bands were homogenized in distilled water, then Instagel Plus solution (Packard, Gröningen, The Netherlands) was added and the radioactivity counted using a TRI-CARB 4000 counter (Packard).

**Western blot analysis**

After SDS-PAGE, proteins were transferred onto PVDF-membranes (Immobilon-P, Millipore, Bedford, MA) according to the method of
Towbin et al. (1979). Then, membranes were saturated by incubation for 2 hours with a 20 mM Tris-HCl buffer, pH 7.4, containing 0.5 M NaCl and 5% (w/v) non-fat dry milk (Bio-Rad, Ivry sur Seine, France) at room temperature, and incubated for 18 hours in the same buffer containing monoclonal or polyclonal antibodies as indicated. Alkaline phosphatase-conjugated goat IgG anti-mouse IgG or alkaline phosphatase-conjugated goat IgG anti-rabbit IgG (Sigma) were used as secondary antibodies (dilution 1:10,000). The reacting proteins were detected by chemiluminescence using the Immuno-Star chemiluminescence kit from Bio-Rad and Hyperfilm™ ECL™ (Amersham).

**Northern- and blot-analysis**

Total RNA was extracted from 15×10^6 thyroid epithelial cells cultivated in polystyrene flasks treated for tissue culture, in 4 M guanidinium isothiocyanate, 25 mM sodium citrate buffer, pH 7.0, containing 0.5% Sarcosyl and 0.1 M 2-mercaptoethanol with agitation for 20 seconds, according to the method of Chomczynski and Sacchi (1987). Then, an equal volume of phenol and 0.2 volume of chloroform/isoamyl alcohol (49/1, v/v) mixture were added in the presence of 0.1 volume of 2 M sodium acetate, pH 4.0. The mixture was stirred, cooled on ice and centrifuged at 10,000 g for 20 minutes. The RNA contained in the aqueous phase was precipitated by addition of 2 volumes of isopropanol. After centrifugation at 10,000 g for 5 minutes, the precipitate was dissolved in water and precipitated again with 75% ethanol solution. The precipitate was finally dissolved in water. Total RNA content was evaluated by A260 measurement and its integrity checked by agarose gel electrophoresis. Standards of 18S and 28S ribosomal RNA were used as control of molecular size. For northern blot analysis, total RNA (6 μg of each sample) was denatured in formamide and electrophoresed in 1.0% (w/v) agarose gels containing 2.2 M formaldehyde (Maniatis et al., 1982). After electrophoresis, RNAs were stained with ethidium bromide to confirm their integrity and transferred overnight by the capillary method in 1× SSC, 5 mM Tris-HCl buffer, containing 2 mM CaCl₂, pH 7.8, and then chromatographed in a linear gradient of NaCl from 300 mM to 2 M. The fraction eluted at 154 mM NaCl and 2 mM CaCl₂, pH 7.6. Platelet aggregation was induced by the addition of 0.5 μM/ml human thrombin (Sigma) for 5 minutes at 37°C. Reaction was stopped with benzamidine 5 mM (Sigma) at 4°C. Aggregated platelets were sedimented at 25000 g for 15 minutes at 4°C and the supernatant was immediately frozen at −80°C. It was thawed at 4°C overnight and then centrifuged at 20000 g for 15 minutes at 4°C.

**TSP-1 purification from porcine platelets**

Porcine blood was obtained from a local slaughterhouse. Blood was mixed with citric acid/citrate/dextrose buffer, pH 6.8 (ACD) (0.8% (w/v) citric acid, 2.2% (w/v) trisodium citrate, 2.45% (w/v) dextrose) at 1 vol. ACD for 6 vol. blood. Platelets were isolated according to the method of Legrand et al. (1984), slightly modified as follows: the blood was centrifuged at 120 g for 15 minutes at 20°C to give platelet-rich plasma, then platelets were sedimented at 1200 g for 15 minutes. They were washed three times in washing buffer consisting of 35 mM citric acid, 154 mM NaCl, 5 mM glucose, 5 mM KCl, 1 mM MgCl₂, 25 μM/ml Apyrase (Sigma) and 100 mM prostaglandin E1 (Sigma), pH 6.5 (centrifugation was performed at 1200 g for 15 minutes). The platelet number was adjusted to 2×10⁹ per ml in 20 mM Tris-HCl, 154 mM NaCl and 2 mM CaCl₂, pH 7.6. Platelet aggregation was induced by the addition of 0.5 mM/ml human thrombin (Sigma) for 5 minutes at 37°C. Reaction was stopped with benzamidine 5 mM (Sigma) at 4°C. Aggregated platelets were sedimented at 25000 g for 15 minutes at 4°C and the supernatant was immediately frozen at −80°C. It was thawed at 4°C overnight and then centrifuged at 20000 g for 15 minutes at 4°C.

The TSP-1 purification process consisted of two steps: the supernatant of aggregated platelets was first chromatographed on a HitTrap Heparin-Sepharose CL-6B column (Pharmacia Biotech, Orsay, France) in 20 mM Tris-HCl buffer containing 300 mM NaCl, 2 mM CaCl₂ and 2 mM benzamidine, pH 7.6. TSP-1 was eluted by a linear gradient of NaCl from 300 mM to 2 M. The fraction eluted at 0.55 M NaCl and containing TSP-1 was dialyzed against 20 mM Tris buffer, containing 2 mM CaCl₂, pH 7.8, and then chromatographed in the same buffer on a Bio-Scale Q 2 column (Bio-Rad) using an FPLC system (Biologic from Bio-Rad). TSP-1 was then eluted by NaCl (0.02 to 1 M linear concentration gradient) and recovered in the fraction eluted at 0.3 M. The recovery was 600 μg TSP-1/μl plasma. The fraction was dialyzed and concentrated against 20 mM Tris-HCl, 154 mM NaCl, 2 mM CaCl₂ (pH 7.6) buffer and the purity of TSP-1 was checked by SDS-PAGE By this method no additional Coomassie blue stained protein contaminants were observed (data not shown). Since it is known that TSP-1 binds and activates latent TGF-β1 (Schultz-Chery et al., 1994), the amount of TGF-β1 bound to our preparation of porcine TSP-1 was determined by a TGF-β1 ELISA System (Promega, France). As the human monoclonal antibody (Sigma) used in this system was known to recognize only the active form of TGF-β1 and to cross-react with porcine TGF-β1, we
performed the assay on non-treated and treated samples by HCl solution (1 N) for 1 minute and neutralized with NaOH solution in order to activate latent TGF-β1. Only latent TGF-β1 was detected in our preparations at a ratio of 2.75 pg/μg TSP-1. We verified that the effects of TSP-1 mentioned in this study were not mediated by the TSP-1-bound TGF-β (not shown).

**Monoclonal and polyclonal antibodies**

Polyclonal antibodies to porcine TSP-1 were produced in rabbits by injecting the unreduced form of porcine TSP-1 purified as described. For this purpose, after SDS-PAGE on a 4.5% (w/v) acrylamide gel and Coomassie blue staining, the band corresponding to the unreduced form of TSP-1 was excised and injected subcutaneously in rabbits. Injections were repeated each week for one month. Then the blood was collected and the reactivity of antiserum tested. The antibodies recognize both the native and reduced forms of TSP-1 by western blot analysis (it was used at 1:1000 dilution). Rabbit polyclonal anti-human integrin αv subunit and anti-human integrin β3 subunit antisera were from Bioproducts, Heidelberg, Germany (species reactivities were shown for human, goat, equine, porcine, sheep and mouse). Monoclonal antibody to human αvβ3 integrin (clone LM609) was purchased from Chemicon (Euromedex, Soufflèweyerheim, France). It also recognizes porcine αvβ3 integrin.

**Cell adhesion and migration**

To examine the adhesive or anti-adhesive property of TSP-1 on follicle cells, freshly isolated thyroid cells were cultured in suspension in bacterial dishes in MEM containing 10% (v/v) FCS and 1 mU/ml TSH, for two days, in order to reorganize cells into follicles. Bacteriological dishes coated or not with TSP-1 or with BSA were prepared as follows: TSP-1 was diluted with 20 mM sodium carbonate buffer, pH 9.6, to a final concentration of 500 μg/ml. Aliquots of 0.1 ml of this solution were deposited at the center of 35 mm-diameter dishes as a spot covering a 1 cm² surface, and kept at 4°C for 18 hours. Some dishes were coated with 100 μg/ml of TSP-1. In some dishes, cyclic RGD- or RGE-containing peptide (100 μg/ml) (Neosystem, Strasbourg, France) or a monoclonal antibody directed against the αvβ3 integrin (2 μg/ml) were added in the presence or in the absence of heparin (100 μg/ml) (Sigma). At the end of the incubation period, the culture dishes were aspirated and washed three times with Earle-Hepes solution. The adherent follicle cells were visualized by phase contrast microscopy.

To examine the influence of TSP-1 on cell behaviour, freshly isolated thyroid cells (2×10⁶ cells/ml) were incubated for two days at 37°C in MEM supplemented with 2% (v/v) FCS and 1 mU/ml TSH, in polystyrene 6-well plates treated for tissue culture (Nunc) or in polystyrene 6-well plates coated with TSP-1 (5 μg/cm²) as described above excepted that TSP-1 covered the surface of each well. In some non-coated wells, soluble TSP-1 (50 μg/ml) was added in the culture medium. Cells were observed by phase contrast microscopy at varying times of culture.

To examine the role of TSP-1 on the reorganization of cells induced by TGF-β1, freshly isolated thyroid cells (2×10⁶ cells/ml) were incubated in MEM supplemented with 2% (v/v) FCS and 1 mU/ml TSH for 2 days at 37°C in tissue culture polystyrene 6-well plates. Then, the medium was changed and cells incubated for 48 hours at 37°C in 1 ml of MEM containing 2% (v/v) FCS, 1 mU/ml TSH, 50 μg/ml ascorbic acid, 50 μg/ml 2-amino-5-oxo-5-phosphoribosyl imidazole, and in the presence of either 2 ng/ml TGF-β1 or in TGF-β1-free medium. In some dishes, cyclic RGD- or RGE-containing peptide (100 μg/ml), heparin (100 μg/ml), monoclonal antibody to the αvβ3 integrin (20 μg/well), rabbit polyclonal antibody to porcine TSP-1 (100 μl antisem per well) or a combination of these was added to the incubation medium. Cells were observed by phase contrast microscopy.

**DNA synthesis measurement**

Proliferation was evaluated by measuring total DNA. For this purpose, one ml of freshly isolated thyroid cells (5×10⁶ cells) were cultured on non-coated or TSP-1-coated (5 μg/cm²) 6-well polystyrene plates in MEM containing 10% (v/v) FCS, 1 mU/ml TSH and in the presence or in the absence of soluble TSP-1 (50 μg/ml). Organization of cells was observed at varying periods of culture by phase contrast microscopy. The adhering cells were harvested by scraping, washed twice in Earle-Hepes buffer (pH 7.2) and sonicated in 50 mM phosphate buffer containing 2 M NaCl and 2 mM EDTA (pH 7.4) in crushed ice. Aliquots of 20 μl were used to quantify DNA using the Hoechst fluorescent reagent (0.2 mg/ml), (Sigma), according to the method of Chrest et al. (1993). In the case of kinetic studies with TGF-β1, freshly isolated thyroid cells were cultured in 6-well polystyrene plates in MEM containing 10% (v/v) FCS for two days in the presence of TSH (1 mU/ml). Then, TGF-β1 was added in fresh medium at a concentration of 2 ng/ml, and cells were incubated for varying periods of time (0 to 48 hours).

**Statistical evaluation**

Quantitative experiments were performed in quadruplicate (except for kinetic experiments performed in duplicate) and results expressed as the mean ± s.d. The statistical significance of the results was studied by Student’s t-test (Snedecor and Cochran, 1967).

**RESULTS**

**Effect of TGF-β1 on cell morphology**

After two days of culture in the presence of TSH (1 mU/ml), thyroid cells were reassociated into follicular structures. In contrast, the addition of TGF-β1 to the medium induced a concentration- and time-dependent antagonist effect on the organizing action of TSH (Fig. 1A and B). In the presence of TGF-β1 follicles progressively disrupted and cells migrated and formed a tight monolayer, sometimes forming domes after 48 hours in the presence of 2 or 10 ng/ml TGF-β1.

**Concentration- and time-dependent effect of TGF-β1 on TSP-1 synthesis**

TGF-β1 stimulated concentration-dependent secretion of TSP-1 by thyroid cells (Fig. 2A). Western blot analysis of TSP-1 under reducing conditions showed a TGF-β1 concentration-dependent increase of TSP-1 (180 kDa) in the medium whereas TSP-1 was not detected in the cell layer Fig. 2A). The amount of Tg secreted in the medium was slightly increased by TGF-β1 (Fig. 2B). However, TGF-β1 induced a concentration-dependent decrease of Tg in the cell layer. The amount of Tg in the cell layer represented about 10% of the total amount of Tg synthesized by thyrocytes and the sum of secreted Tg + cell Tg remained constant (data not shown). This observation could be related to the progressive disruption of follicles elicited by TGF-β1. No difference in the effect of TGF-β1 was observed between cells incubated from day 0 to day 2 of culture and cells incubated from day 2 to day 4 of culture (data not shown).

TSP-1 was increased in the culture medium as early as after 4 hours of incubation with 2 ng/ml TGF-β1 and progressively...
Effect of TGF-β1 on thyroid cells

reached a maximum after 48 hours incubation (Fig. 2C). The effect of TGF-β1 (2 ng/ml) on the expression of TSP-1 was also confirmed by northern blot analysis (Fig. 3). The steady-state level of TSP-1 mRNA progressively increased up to 24 hours incubation whereas that of thyroglobulin mRNA was not modified. In contrast, the steady-state level of GAPDH mRNA
decreased with the incubation time in the presence of TGF-β1 and excluded the use of GAPDH gene expression as internal control in these experiments. However, RNA ethidium bromide staining showed that similar amounts of total RNA were analyzed. When follicle cells were incubated in the presence of anisomycin, an inhibitor of protein synthesis, the amount of labeled polypeptides secreted into the medium strongly decreased, both in the absence or presence of TGF-β1, and cells maintained their follicle-like structure (data not shown).

Fig. 2. Concentration- and time-dependent effect of TGF-β1 on TSP-1 and Tg synthesis in porcine thyroid cells in culture. Freshly isolated thyroid cells (3x10^6 cells/ml) were cultured for two days in the presence of 1 mU/ml TSH and 10% (v/v) FCS, as described in Fig. 1, and then incubated in fresh medium supplemented with 2-aminopropionitrile fumarate (50 µg/ml), ascorbic acid (50 µg/ml), 2% (v/v) FCS, 1 mU/ml TSH and [35S]methionine (10 µCi/ml). Human recombinant TGF-β1 was added to the incubation medium either at varying concentrations for 48 hours (A and B) or 2 ng/ml for varying periods of incubation (C). At the end of the incubation period, the medium and the cell layer were recovered, dialyzed and lyophilized and then analyzed by SDS-PAGE and western blot as described. (A) Western blot analysis of TSP-1 from the medium and the cell layer. Samples (200 µg lyophilized material) from the medium and the cell layer were subjected to SDS-PAGE on a 7.5% (w/v) acrylamide gel under reducing conditions and then transferred to a nylon membrane. TSP-1 was revealed by rabbit polyclonal antibodies to porcine TSP-1 (dilution 1:1,000) and alkaline phosphatase-conjugated goat IgG anti-rabbit IgG antibodies (dilution 1:10,0000) followed by chemiluminescence detection using the Immun-Star chemiluminescence kit from Bio-Rad. (B) Tg synthesis was studied by metabolic incorporation of [35S]methionine (20 µCi/ml) for 48 hours followed by SDS-PAGE analysis with 4.5% (w/v) acrylamide of samples (200 µg lyophilized material) from the medium and the cell layer under reducing conditions. After electrophoresis, the gel was processed for fluorography as described. (C) Accumulation of Tg and TSP-1 in the culture medium in the presence of TGF-β1. Tg and TSP-1 were analyzed after metabolic incorporation of [35S]methionine (20 µCi/ml) for varying periods of incubation by SDS-PAGE with 4.5% (w/v) acrylamide under non-reducing and reducing conditions. After electrophoresis, the gel was processed for fluorography as described. No TSP-1 was seen in the culture medium of cells incubated for 48 hours in the absence of TGF-β1 (see A, lane 0).

Role of TSP-1 in cell morphology and cell proliferation
To examine the effect of TSP-1 on cell reorganization induced by TGF-β1, we first studied the adhesive property of TSP-1 for follicle cells (Fig. 4). Adhesion of cells was observed on a TSP-1-coated surface and was inhibited by a RGD-containing peptide in association with heparin or by a monoclonal antibody to the αvβ3 integrin also in combination with heparin. When cells were plated on polystyrene surfaces (plastic surface) in the presence of soluble TSP-1 or on TSP-
Fig. 3. Northern blot analysis of total mRNA extracted from thyroid cells incubated with 2 ng/ml TGF-β1 for varying periods of incubation. Thyroid cells ($15 \times 10^6$ cells/flask) organized into follicle-like structures with 1 μM TSH for two days culture were incubated in fresh MEM containing 10% FCS, 1 μM TSH and 2 ng/ml TGF-β1 for varying periods. At the end of the incubation period total RNA was extracted and aliquots corresponding to 6 μg RNA were electrophoresed on 1% (w/v) agarose gels and then transferred to nylon membranes. Sequential hybridizations of mRNA blotted on membranes were performed with THG, TSP-1 and GAPDH [32P]cDNA probes and processed for autoradiography as described (A). The integrity and the amount of RNA loaded on 1% (w/v) agarose gels were checked before and after blotting (B) by ethidium bromide staining.

1-coated plastic surfaces, they progressively spread and migrate instead of forming follicular structures as observed on plastic substratum only (Fig. 5A). This effect was abolished by a cyclic RGD-containing peptide (Fig. 5B). In the absence of TGF-β1, the peptide induced complete cell detachment from the plastic substratum and cells organized into floating follicles whereas on TSP-1-coated surfaces, RGD inhibited migration and cells organized into follicles which remained attached to the substratum. The addition of RGD peptide into the medium also prevented the effect of TGF-β1 on cell behaviour and cells remained as a follicle-like structure attached to the substratum as on a TSP-1-coated surface.

Possible involvement of TSP-1 and αvβ3 integrin in the transition of thyroid cells from follicle-like structures to monolayer

To examine the presence of TSP-1 receptors on thyroid cells, western blot analysis of extracts of thyroid cells incubated with varying concentrations of TGF-β1 was performed using polyclonal antibodies to either the αv or the β3 integrin subunit. As shown in Fig. 6, TGF-β1 induced a concentration-dependent increase in the amount of both the αv and β3 integrin subunits. As shown in Fig. 7, when added to the culture medium of follicle cells, monoclonal antibody to the αvβ3 integrin partly prevented the effect of TGF-β1 on cell spreading and migration. Polyclonal antibodies to TSP-1 also abolished the effect of TGF-β1 on cell spreading and migration but they seemed to exert less inhibitory action than anti-αvβ3 antibody. Heparin alone slightly affected TGF-β1-induced cell spreading and migration but seemed to act in synergy with the respective antibodies when it was added in combination with them.

**Table 1. Effect of TGF-β1 and TSP-1 on cell proliferation**

<table>
<thead>
<tr>
<th>Time of incubation (hours)</th>
<th>A - mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>TGF-β1</td>
<td>- TSP-1</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>99</td>
</tr>
<tr>
<td>4</td>
<td>100</td>
</tr>
<tr>
<td>8</td>
<td>98</td>
</tr>
<tr>
<td>16</td>
<td>97</td>
</tr>
<tr>
<td>24</td>
<td>92</td>
</tr>
<tr>
<td>48</td>
<td>94</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>+ TSP-1</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>88</td>
</tr>
<tr>
<td>4</td>
<td>78</td>
</tr>
<tr>
<td>8</td>
<td>89</td>
</tr>
<tr>
<td>16</td>
<td>76</td>
</tr>
<tr>
<td>24</td>
<td>71</td>
</tr>
<tr>
<td>48</td>
<td>73</td>
</tr>
</tbody>
</table>

TGF-β1 experiments (A) were processed as follows: freshly isolated porcine thyroid cells ($3 \times 10^6$ cells/ml) were cultured in MEM containing 10% (v/v) FCS and 1 μM TSH for 2 days at 37°C in 25 cm² polystyrene flasks treated for tissue culture. Then, the medium was replaced by fresh MEM containing 10% (v/v) FCS, 1 μM TSH and no or 2 ng/ml human recombinant TGF-β1, and cells were incubated for varying periods at 37°C.

In experiments with TSP-1 (B), follicle cells were cultured in TGF-β1 free medium in polystyrene flasks and in the presence or absence of soluble TSP-1 (50 μg/ml) or on TSP-1-coated flasks (5 μg/cm²). The amount of DNA was measured at the end of each period of incubation by a fluorescent method using Hoechst reagent. The results were expressed either as a percentage of control (To) (A) or in μg DNA per flask (B).
DISCUSSION

Since TGF-β1 was shown to be produced by the rat thyroid cell line FRTL-5 (Morris et al., 1988) and detected in human thyroid follicular cells (Grübeck-Lobenstein et al., 1989), its role as a local modulator of thyroid function has been advanced. In the present work, we studied the effect of TGF-β1 on the behaviour of porcine thyroid cells cultured on a conventional adhesive substratum in order to define its influence on a cellular system in which cells are forming follicle-like structures.

We observed that the stimulation of follicles with TGF-β1 for 48 hours, either at the beginning of the culture or after a two day culture in the presence of TSH promoted an important modification in cell organization. TGF-β1 was able to promote the disintegration of TSH-induced follicles. TGF-β1 induced a flattened cellular phenotype with spreaded cells remaining attached to the flasks. This morphological change is in agreement with previous studies and can be due to the modification of cytoskeleton as already reported in the FRTL-5 cell line (Nilsson et al., 1995; Taton et al., 1993) and/or to the loss of cell polarization as shown in porcine thyrocytes embedded in collagen gel (Toda et al., 1997).

The transition from follicular to monolayer state induced by TGF-β1 was not accompanied by cell proliferation but, on the contrary, by a moderate decrease of DNA content in follicle cells. This result is in agreement with previous studies reporting an inhibitory effect of TGF-β1 on cell growth and/or DNA synthesis (see Introduction). As suggested by Nilsson et al. (1995), mitogenic and migratory signals can be regulated independently. The inhibition by TGF-β of the TSH/cAMP-dependent synthesis of PCNA (proliferating cell nuclear antigen)/cyclin which is necessary for DNA synthesis could account for its effects on DNA synthesis (Taton et al., 1993).

The most relevant indexes of differentiation state in thyroid cells in primary cultures are constituted by the ability of cells to concentrate and organify iodide and to respond to acute TSH stimulation that may also depend on the cell organization. TGF-β1 strongly decreased 125I-protein bound iodide under long term conditions even though we could not observe any
modulation of iodide organification with a TGF-β1 acute stimulation (data not shown). This inhibition was not related to a decrease of thyroglobulin gene expression as was reported in other studies (Colletta et al., 1989) but was due to an alteration of the NADPH oxidase-thyroperoxidase system coupled to an inhibition of iodide trapping (data not shown) or to the disorganization of follicle cells. The inhibition by TGF-β1 of the TSH-induced stimulation of iodide uptake and thyroperoxidase synthesis was already reported (Coletta et al., 1989; Pang et al., 1992; Taton et al., 1993). We also found that TGF-β1 inhibits the cAMP-responsiveness of porcine thyroid cells to TSH, suggesting that it is also able to interfere with the

Fig. 5. RGD peptide prevented the effect of both TSP-1 and TGF-β1 on cell disruption and migration. (A) Freshly isolated thyroid cells (2×10^6 cells/ml) were cultured in MEM containing 2% (v/v) FCS and 1 mU/ml TSH in polystyrene plates treated for tissue culture (plastic substratum) in the presence or absence of soluble TSP-1 (50 μg/ml) (soluble TSP-1) or into polystyrene plates coated with TSP-1 (5 μg/cm²) (coated TSP-1). Cells were observed by phase contrast microscopy. TSP-1 induced significant cell adhesion and spreading. Cells progressively migrate and reach confluency after a 48 hour incubation period both on TSP-1-coated dishes and on a plastic surface in the presence of soluble TSP-1 whereas they formed follicle-like structures on a plastic substratum in TSP-1-free cultures. Bar, 70 μm. (B) Freshly isolated cells (2×10^6 cells/ml) were cultured in MEM containing 2% (v/v) FCS and 1 mU/ml TSH in polystyrene 6-well plates coated or not with TSP-1 (5 μg/cm²) for two days at 37°C. Cells were then incubated in one ml of fresh medium supplemented with 2% (v/v) FCS, 1 mU/ml TSH, 50 μg/ml ascorbic acid, 50 μg/ml 2-aminopropionitrile fumarate and in the absence or presence of TGF-β1 (2 ng/ml) for 2 days. Cyclic RGD- or RGE-containing peptide was also added to the incubation medium of some wells at 100 μg/ml concentration. Morphology of cells was observed by phase contrast micrography. Bar, 40 μm.
cAMP signal pathway (data not shown) at variance with its effect on human thyroid cells (Taton et al., 1993).

Another important aspect of the role of TGF-β1 in the regulation of thyroid functions concerns the relationship between the activity of TGF-β1 as a regulator of cell growth and differentiation and its effects on the expression of extracellular matrix components. We demonstrated, as in other cell systems (Negoescu et al., 1995), that TGF-β1 stimulated the expression of the αvβ3 integrin. Freshly isolated thyroid cells (3×10⁶ cells per ml) were cultured for two days in MEM containing 10% (v/v) FCS and 1 mU/ml TSH in 25 cm² culture flasks. Cells were then incubated in fresh medium supplemented with 2% FCS, 1 mU/ml TSH and varying concentrations of TGF-β1 for 48 hours. The medium was removed and the cell layer washed twice in saline solution. Similar aliquots of cell extracts in 2% SDS, 10% glycerol, 25 mM Tris-HCl, pH 6.8, were subjected to SDS-PAGE on a linear gradient (4-10%) polyacrylamide gel and immunoblotting analysis. The αv and β3 integrin subunits were revealed by a chemiluminescent detection system using rabbit polyclonal antibodies to the αv and β3 integrin subunits (dilution 1:1000). Alkaline phosphatase conjugated goat IgG anti-rabbit IgG (dilution 1:1000) was used as secondary antibody and CDP-star chemiluminescent substrate as revelation system. Molecular masses are indicated in kDa.

Fig. 6. TGF-β1 increased the expression of the αvβ3 integrin. Freshly isolated thyroid cells (3×10⁶ cells per ml) were cultured for two days in MEM containing 10% (v/v) FCS and 1 mU/ml TSH in 25 cm² culture flasks. Cells were then incubated in fresh medium supplemented with 2% FCS, 1 mU/ml TSH and varying concentrations of TGF-β1 for 48 hours. The medium was removed and the cell layer washed twice in saline solution. Similar aliquots of cell extracts in 2% SDS, 10% glycerol, 25 mM Tris-HCl, pH 6.8, were subjected to SDS-PAGE on a linear gradient (4-10%) polyacrylamide gel and immunoblotting analysis. The αv and β3 integrin subunits were revealed by a chemiluminescent detection system using rabbit polyclonal antibodies to the αv and β3 integrin subunits (dilution 1:1000). Alkaline phosphatase conjugated goat IgG anti-rabbit IgG (dilution 1:1000) was used as secondary antibody and CDP-star chemiluminescent substrate as revelation system. Molecular masses are indicated in kDa.

Fig. 7. Involvement of TSP-1 and αvβ3 integrin in cell spreading and migration induced by TGF-β1. Freshly isolated thyroid cells (2×10⁶ cells per ml) were cultured for two days in MEM containing 10% (v/v) FCS and 1 mU/ml TSH in polystyrene 6-well culture plates. Follicles were then incubated for 48 hours in one ml of fresh medium supplemented with 2% (v/v) FCS, 1 mU/ml TSH, 2 ng/ml TGF-β1, 50 μg/ml 2-aminopropionitrile fumarate and 50 μg/ml ascorbic acid. A monoclonal antibody to the αvβ3 integrin (20 μg IgG/ml), polyclonal antibodies to TSP-1 (100 μl antiserum/ml) and heparin (100 μg/ml) were added alone or in combination in the medium at the beginning of the incubation period. Morphology of cells was observed by phase contrast microscopy and compared to cells cultured in the absence of TGF-β1. Bar, 100 μm.
strongly the synthesis of TSP-1 in a concentration-dependent way since the steady-state level of TSP-1 mRNA as well as the protein amount increased. On the other hand, it seemed that TGF-β1 counteracted the effect of TSH on GAPDH expression since the steady-state level of GAPDH mRNA decreased. It was already reported that mitogenic factors like TSH, TPA or EGF increased levels of GAPDH mRNA in primary cultured thyrocytes (Savonet et al., 1997). The effect of TGF-β1 that we observed could be related to its anti-mitogenic action on thyroid cells as already discussed and not to differences in the amount of RNA analyzed. Furthermore, we observed that porcine thyroid cells cultured either on TSP-1-coated dishes or in the presence of soluble TSP-1 added to the culture medium exhibited an organization similar to that induced by TGF-β1. So, it is tempting to speculate that the cell organization changes promoted by TGF-β1 are mediated through TSP-1 synthesis. This assumption is supported by the fact that anisomycin, an inhibitor of protein synthesis, inhibited the effect of TGF-β1 on cell morphology though we cannot excluded the involvement of other extracellular matrix components which have been already reported to affect the organization and function of thyroid cells (Toda et al., 1995) or intracellular regulation of the TGF-β1 transduction pathway. In addition, we have shown that TSP-1 functioned as a thyrocyte adhesive protein probably through its RGDa sequence and N-terminal heparin binding domain since a cyclic RGD-containing peptide or a monoclonal antibody directed against the αβ3 integrin in association with heparin strongly inhibited thyroid cell adhesion. The RGD peptide also stabilized the follicular structures suggesting that TSP-1 can play a role in the disruption of follicle-like structures. However, we did not exclude the possible involvement of receptors like CD36 or CD47 which have been previously reported to bind TSP-1 (Asch, 1993; Gao et al., 1996) or cell interactions with other components of extracellular matrix as known to be expressed by TGF-β1 in different cell systems (Shi et al., 1990; Rifkin et al., 1993). It may explain why cells incubated with TGF-β1 remained attached to a TSP-1-coated surface or to the plastic substratum when, in the absence of TGF-β1, they detached from the plastic substratum. We can hypothesize that RGD promotes cell detachment from the plastic substratum because follicle-cells do not express TSP-1 and only a small amount of other extracellular matrix proteins whereas on TSP-1 substratum, as two distinct adhesion sites are involved, RGD alone is not able to inhibit the cell adhesion. On the other hand, like TGF-β1, TSP-1 also strongly decreased 125I-protein bound iodide but, in contrast, did not inhibit the cAMP-responsiveness of cells to TSH (data not shown). We also observed that RGD peptide maintained the functional activities of thyroid cells treated or not with TSP-1 (i.e. cAMP responsiveness of cells to TSH and protein iodination) like those obtained with cells organized into a follicle-like structure whereas RGD reversed only the inhibitory effect of TGF-β1 on protein iodination (unpublished results). The characterization of TSP-1 as an adhesive protein for thyroid cells is in agreement with data reported in the literature with different systems (Tuszynski et al., 1987; Pellerin et al., 1993; Varani et al., 1988; Frazier, 1987). Nevertheless, TSP-1 in adrenocortical cells was found to be efficient for cell attachment to plastic but to have no influence on cell spreading (Pellerin et al., 1994). In our model, the mechanism by which TSP-1 exerts its effects on thyroid cell spreading and migration has to be further studied at the cell level. We only show that TSP-1 like TGF-β1 failed to stimulate cell proliferation. As mentioned by Bornstein (1995), no evidence was reported for a role of TSP-1, either alone or in concert with growth factors, in cell proliferation.

The storage in the extracellular matrix of factors like TGF-β1 and the potential activation of TSP-1 synthesis are extremely interesting for the understanding of the mechanisms involved during pathological processes. The extracellular matrix components and particularly TSP-1 must be regarded as active players in the regulation of thyroid cell function. Further studies on the relationships between TGF-β1 and TSP-1 in porcine thyroid cells could produce information of primary interest about the negative feedback triggered by these molecules on the TSH-dependent metabolic pathways.

This work was supported by a grant from the ARC (Association pour la Recherche contre le Cancer, no. 6580) and by CNRS. We warmly thank the people at the slaughterhouse of Rethel (SOBEVIR) who provided us with pig thyroid glands. We thank Mrs O. Legue for her expert technical assistance.

REFERENCES


