Release of an Endothelial Cell Growth Factor from Cultured Porcine Thyroid Follicles

W. Greil, M. Rafferzeder, G. Bechtner, and R. Gärtner

Medizinische Klinik Innenstadt der Universität München
D-8000 München 2, Federal Republic of Germany

It has been proposed from *in vivo* studies that thyroid angiogenesis during thyroid enlargement may be due to paracrine mitogenic factors released by epithelial thyroid cells. To study this paracrine growth regulating communication between thyroid cells and endothelial cells *in vitro*, culture medium from isolated porcine thyroid follicles was investigated for a growth promoting effect on porcine aortal endothelial cells. Serum-free conditioned medium (CM) from thyroid follicles in suspension culture contains a dose-related mitogenic activity which stimulates endothelial cell growth up to 197%. Stimulation of the thyroid follicles with TSH (1 mU/ml) significantly reduced the mitogenic activity for endothelial cells in CM to 131%. Thyroid hormones had no influence on mitogenic activity in CM. When follicles were treated with iodide (20 μM) during CM production, no proliferation of endothelial cells was observed by this CM. In contrast, CM from epidermal growth factor-treated thyroid follicles significantly enhanced the mitogenic activity for endothelial cells up to 235%. The mitogenic activity was precipitable by saturated ammonium sulfate, showed high affinity to heparin by chromatography on heparin-sepharose, and was abolished after treatment of CM with trypsin. On gel electrophoresis the heparin-binding fraction showed a double band with a mol wt of 15 and 15.5 k. These data show a paracrine mitogenic activity on endothelial cells released by thyroid follicles which is regulated by TSH, epidermal growth factor, and iodide in parallel with the direct effect of these substances on thyroid cell growth. The data suggest that the mitogenic factor is a polypeptide, which belongs to the heparin-binding growth factors. (Molecular Endocrinology 3: 858–867, 1989)

INTRODUCTION

Mesenchymal cells adapt to the changing requirements in growing tissues. It has become a general accepted principle that this concomitant growth of the different cells is mediated by paracrine growth factors. These growth factors are responsible for the simultaneous stimulation of growth of organ specific cells, connective tissue, and blood vessels (1).

A paracrine communication has been shown to be involved not only in the regulation of cellular growth, but also of the specific functions in a wide variety of organs and tissues, *e.g.* in the pituitary gland, in islet cells of the pancreas, in the mammary gland, the ovary, and in the testes (2). In particular, the paracrine regulation involving growth of blood vessels plays an important role in follicle maturation in the ovary (3, 4) and in the formation of the corpus luteum (5). In growing tumor tissue it has been shown, that a paracrine factor (tumor angiogenesis factor) is released from the tumor cells which induced growth of capillary vessels (6).

During goiter genesis in rats an enlargement of vessel diameters and a peak of ^{3}H-thymidine labeling in capillaries, veins, arteries, and lymphatic vessels within and next to the thyroid gland 2 days after induction of goiter genesis could be demonstrated (7, 8). Also in mice under low iodine diet and propylthiouracil treatment it could be shown that endothelial cell proliferation preceded thyroid cell proliferation: whereas endothelial cells started to grow 4 days after the beginning of goiter induction, thyrocytes proliferated with a delay of 8 days (9). Therefore a paracrine factor that is produced by thyrocytes and stimulates growth of endothelial cells was postulated (7–9).

Recently we have demonstrated a mitogenic activity in conditioned medium (CM) from isolated porcine thyroid follicles stimulating cell growth of cultured human fibroblasts (10–12). Here we report that this CM also contains mitogenic activity for endothelial cells. This mitogenic activity is due to a mitogenic factor, which binds to heparin-sepharose, is labile to protease treatment and appears on sodium dodecyl sulfate (SDS)-gelelectrophoresis as a double band with an apparent mol wt of about 15 k. Incubation of thyroid follicles with TSH reduced the mitogenic activity in CM, whereas epidermal growth factor (EGF) increased it. After supplementation of the follicles with iodide no mitogenic activity in CM was found. These results are in parallel with the direct effects of these substances on cell growth of isolated porcine thyroid follicles and may in

0888-8809/89/0858-0867$02.00/0
Molecular Endocrinology

part contribute to the better understanding of thyroid angiogenesis and growth regulation of endemic goiter.

RESULTS

Growth Experiments with Isolated Porcine Thyroid Follicles

Isolated thyroid follicles in suspension are shown in Fig. 1. The follicles are free of contaminating single cells and have a preserved polarity for more than 6 days in culture as shown previously (13).

When the follicles were seeded on hydrophilic culture dishes coated with gelatine, a dose-dependent and significant increase of cell numbers was found after stimulation with EGF (1–5 ng/ml) up to 278 ± 85.8%, with insulin-like growth factor-I (IGF-I, 100 ng/ml) up to 132 ± 7% and with fetal calf serum (FCS) (1–5%) (vol/vol) up to 272 ± 97.1% compared to controls. No change of cell numbers was found with TSH up to 0.1 mU/ml, but with 1 mU/ml TSH a significant inhibition of cell growth was found (83.7 ± 11.8% of control). Basic fibroblast growth factor [(bFGF) 25–100 ng/ml] did not stimulate cell growth (Fig. 2). When the follicles were kept in suspension on hydrophobic culture dishes, no change of cell numbers occurred within 3 days when incubated either with TSH or EGF compared to control.

Response of Endothelial Cell Growth to Specific Growth Factors

Under basal conditions with 2% FCS endothelial cell number was increased within 5 days by about 5-fold. All growth factors were tested in the presence of 2% FCS. Endothelial cell growth supplement [(ECGS] 10–200 μg/ml] stimulated cell growth up to 310 ± 35.3%. Basic FGF (0.1–100 ng/ml) dose dependently stimulated cell growth up to 160 ± 19.3% at 100 ng/ml, higher concentrations did not increase the effect. EGF (1–25 ng/ml) did not affect endothelial cell growth (Fig. 3). Increasing amounts of FCS (5–10%) (vol/vol) stimulated cell growth dose dependently up to 267 ± 79.7% ($n = 9$, $P < 0.01$). Like EGF, IGF-I (1–100 ng/ml) did not stimulate endothelial cell growth (data not shown).

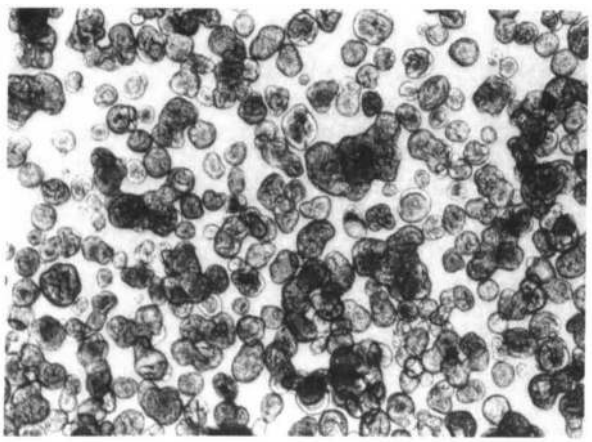

Fig. 1. Light Microscopy of Isolated Porcine Thyroid Follicles after 2 days in Suspension Culture (×40)

Stimulation of Endothelial Cell Growth with CM from Thyroid Follicles

Endothelial cell growth was stimulated dose dependently (10%, 20%, 50%) (vol/vol) by CM from isolated porcine thyroid follicles up to 197 ± 42.9% with 50% (vol/vol) CM compared to control (100 ± 7.54%) (Fig. 4). A 5-fold concentrated CM stimulated cell growth up to 260 ± 8.6% (see Fig. 7). Fresh medium which had gone through all the steps of concentrating, had no stimulatory effect (data not shown).

When endothelial cells were incubated with TSH-CM, obtained from TSH-treated follicles, the cell number of endothelial cells was only increased up to 131 ± 21.3% at a concentration of 50% (vol/vol) CM. The difference between the growth-promoting effects of CM from untreated follicles to TSH-CM is significant (Fig. 5). To see whether the observed reduction of mitogenic activity in TSH-CM is due to increased thyroid hormone levels during TSH stimulation, we tested the influence of T_3 and T_4 on basal mitogenic activity in CM after incubation of thyroid follicles with T_3 or T_4 to produce T_3-CM or T_4-CM. As shown in Table 1 neither T_3-CM nor T_4-CM had any significant effect on the mitogenic activity in the basal CM.

EGF-CM (50%) (vol/vol) obtained from EGF-treated follicles stimulated endothelial cell growth up to 235 ± 22.9%. This increase of growth stimulation by EGF-CM was significant compared to CM without additions and to TSH-CM (Fig. 5).

After incubation of the thyroid follicles with potassium iodide (20 μM), the resulting iodide-CM had a slight inhibitory effect on endothelial cell growth to 80 ± 5.47%, which was not significant compared to controls (Fig. 5). Potassium iodide had no direct influence on endothelial cell growth.

To exclude that the known thyroid secretory products T_3, T_4, thyroglobulin (Tg), and cAMP, which are secreted by thyroid follicles especially after stimulation with TSH (13), are responsible for the growth-promoting activity in CM, we measured the concentrations of these products both in CM and TSH-CM and tested their effects on cell growth of endothelial cells. CM or TSH-CM contained 1.2–1.9 ng/ml T_3, 8–16 ng/ml T_4, 2–500 nM cAMP, and 100–800 ng/ml Tg. Neither T_3 (0.1–5 ng/ml), nor T_4 (1–50 ng/ml), nor cAMP (100–10,000 nM), nor porcine Tg (10–500 ng/ml), or TSH (100–500 μU/ml) stimulated growth of endothelial cells (Fig. 6).

Neither EGF nor IGF-I could be detected in untreated or 5-fold concentrated CM by RRA or RIA, respectively.

Physicochemical Characterization of the Mitogenic Activity in CM

The growth-stimulating effect of CM (50%) (vol/vol) on endothelial cells was preserved after freezing (220 ±

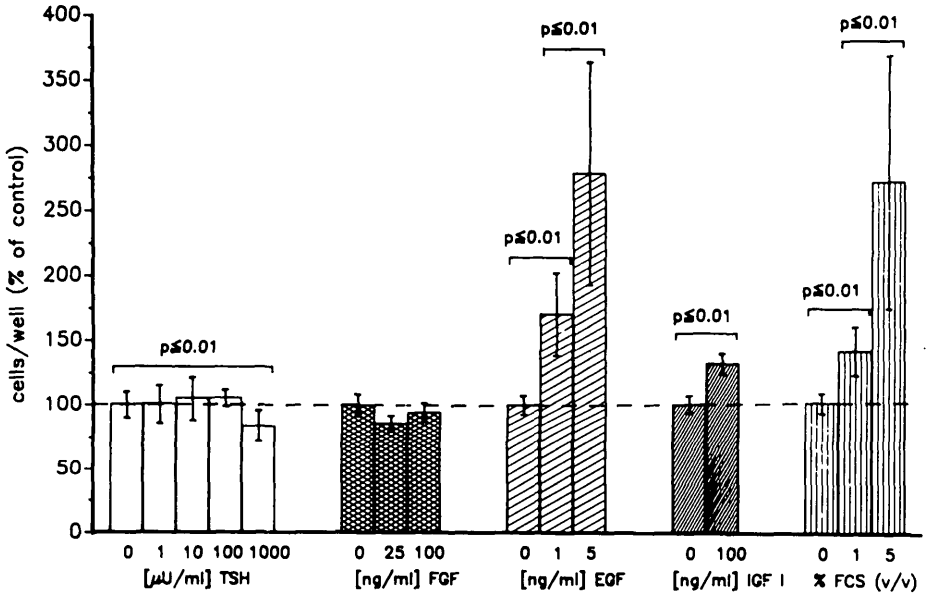

Fig. 2. Growth Experiments with Isolated Porcine Thyroid Follicles
Follicles were stimulated with TSH (n = 9), bFGF (n = 4), EGF (n = 38), IGF-I (n = 8), or FCS (n = 27) as indicated.

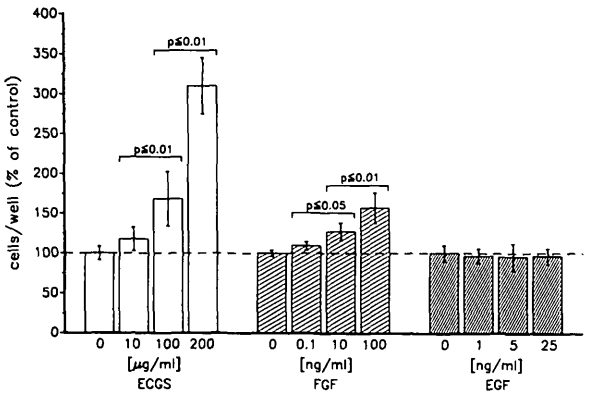

Fig. 3. Growth Stimulation of Endothelial Cells with Growth Factors
Endothelial cells were stimulated with ECGS (n = 6), bFGF (n = 10), and EGF (n = 10) with the respective concentrations.

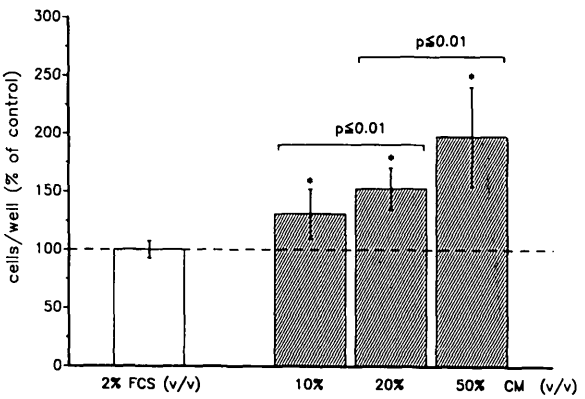

Fig. 4. Growth Stimulation of Endothelial Cells with CM from Isolated Thyroid Follicles
Endothelial cells were incubated with increasing concentrations of CM (10%–50%) (vol/vol) (n = 20–30). *, *P* 0.01, compared to control.

5.29%) or alkaline treatment (231 ± 15.5%) but it was abolished after boiling for 10 min or by acid treatment (Fig. 7). Also treatment of unconcentrated CM with trypsin (10 μg/ml) abolished the mitogenic activity; fresh medium which was used for control and treated in the same manner, had no effect on endothelial cell growth (Table 2).

After precipitation of a 5-fold concentrated CM with 1.9 M ammonium sulfate no mitogenic activity for endothelial cells was found in the pellet. However, the fraction obtained after precipitation with 3.8 M ammonium sulfate stimulated cell growth up to 161 ± 16.0%. In comparison with the 5-fold concentrated CM (50%) (vol/vol) which stimulates cell growth up to 262 ± 8.62%, 40% of the stimulatory activity were recovered in the 3.8 M ammonium sulfate fraction (Fig. 8). After chromatography on heparin-sepharose, the heparin-binding fraction stimulated endothelial cell growth up to 243 ± 45.0% ($P < 0.01$ *vs.* control), when tested at a concentration equal to a 5-fold concentrated, unfractionated CM. The nonheparin-binding fraction did not influence cell growth (Fig. 8).

Analysis of the different fractions from heparin-sepharose by SDS polyacrylamide gel electrophoresis is shown in Fig. 9. Most of the proteins were eluted with the 0.6 M NaCl-fraction (lane A) which had no mitogenic activity. However, in the heparin-binding fraction eluted with 2 M NaCl, two bands were observed: one band which appeared as a doublet with an apparent mol wt of 15 and 15.5 k, and another band with an apparent mol wt of about 60 k (lane C). In the 1 M NaCl fraction

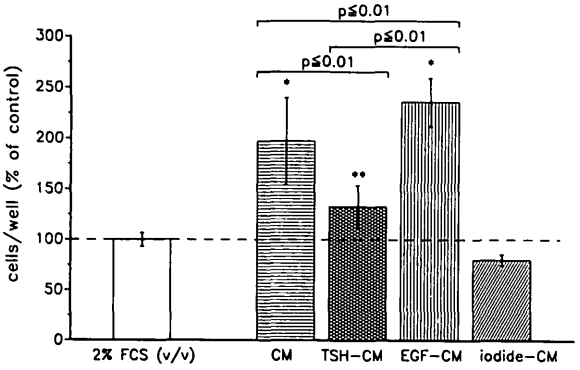

Fig. 5. Influence of TSH, EGF, and Iodide on Mitogenic Activity in CM

Thyroid follicles were incubated either without additions or with TSH (1 mU/ml), EGF (5 ng/ml); or iodide (20 μM). The CM (n = 20; TSH-CM, n = 8; EGF-CM, n = 10; iodide-CM, n = 4) were tested for mitogenic activity on endothelial cells (50%) (v/v). *, $P < 0.01$, **, $P < 0.05$ compared to control.

Table 1. Influence of Thyroid Hormones on Mitogenic Activity in CM

	Cells/Well (% of Control)	
CM	134.2 ± 1.5	
T_3-CM	130.7 ± 7.5	NS
T_4-CM	130.9 ± 6.5	NS

Endothelial cell growth was stimulated with CM, with CM from thyroid follicles incubated either with T_3 (1.5 ng/ml, T_3-CM), or with T_4 (50 ng/ml, T_4-CM), (n=6, mean ± SD, 50%) (vol/vol); NS, not significant from CM.

obviously no proteins could be detected on gel electrophoresis (lane B).

DISCUSSION

Cell Culture Systems

We describe here that intact thyroid follicles release a paracrine factor which stimulates the growth of endothelial cells. The isolated porcine thyroid follicles are well defined concerning their intact morphology, specific function (13), and growth characteristics (14). Endothelial cells from porcine aorta were characterized by their ability to produce angiotensin converting enzyme and prostaglandins. A ratio of 6:1 comparing angiotensin converting enzyme activities of cell medium with cell lysates was found, which is in good agreement with the findings in hog endothelial cell cultures (15). Prostaglandin (PG) production by endothelial cells revealed a characteristical pattern including PGE_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$, as described by Ody *et al.* (16).

For investigation of normal growth potency of endothelial cell cultures, different growth factors were tested. Whereas ECGS, a crude preparation of endothelial cell growth factor which is considered as aFGF (17), and bFGF stimulated endothelial cell proliferation, no mitogenic effect was seen with EGF or IGF-I which is in agreement with others (18–21).

Mitogenic Activity for Endothelial Cells in CM from Thyroid Follicles

Serum-free CM obtained from thyroid follicles in suspension culture dose dependently and significantly increased endothelial cell proliferation. This mitogenic effect was further increased when CM was concentrated by desalting and lyophilization. It has been excluded in control experiments that the proliferation of endothelial cells is due to thyroid hormones, Tg, or cAMP in concentrations comparable to those found in different CMs. It has been suggested that cell growth of cultured GC cells, a T_3-responsive GH-secreting cell line, may be due to secretion of an autocrine growth factor which is induced by T_3 (22). However, in our endothelial cell culture T_3 had no effect on cell proliferation.

Since the mitogenic activity is stable to freezing and alkaline treatment, labile to heat and acid treatment, precipitable by saturated ammonium sulfate, and sensitive to protease treatment, we assume that this growth-promoting activity is related to one or more polypeptide(s). Furthermore, the mitogenic activity shows high affinity for heparin indicating that it belongs to the heparin-binding growth factors (23). On SDS-gel electrophoresis the heparin-binding fraction revealed two bands, one doublet with a mol wt of 15 and 15.5 k and another protein with a mol wt of about 60 k. This double band is very similar to aFGF, which has mol wts of 15.2 and 15.9 k, as deduced from amino acid analysis (17). Although the mitogenic factor found in CM has not yet been identified, these data suggest that the factor may be related to FGF or a FGF-like protein. In other endocrine organs like the adrenal gland, the testes or the ovary, the organ-specific cells also produce FGF or FGF-like activity both *in vitro* and *in vivo* (24–26).

Whereas this is the first demonstration of a paracrine factor with growth-promoting activity on endothelial cells in CM of thyroid follicles, an endotheliotropic chemoattractant activity in conditioned medium from rat thyroid cells has recently been described (27). Also growth-promoting activity for fibroblasts in CM from ovine thyroid cells (28) and from porcine thyroid follicles (10–12) has been previously described. This growth-promoting activity was not related to plasminogen activator, which was found in the CM, since trypsin-inhibitor did not block the growth promoting activity (28). The presence of high levels of IGF-I and II in CM of ovine thyroid cells has also been reported (29). We were not able to detect IGF-I immunoreactivity in CM from porcine thyroid follicles using a sensitive RIA for human IGF-I which has more than 90% cross-reactivity to porcine IGF-I (personal communication from Nichols Institute of Diagnostics).

The activity of the paracrine factor in CM from thyroid

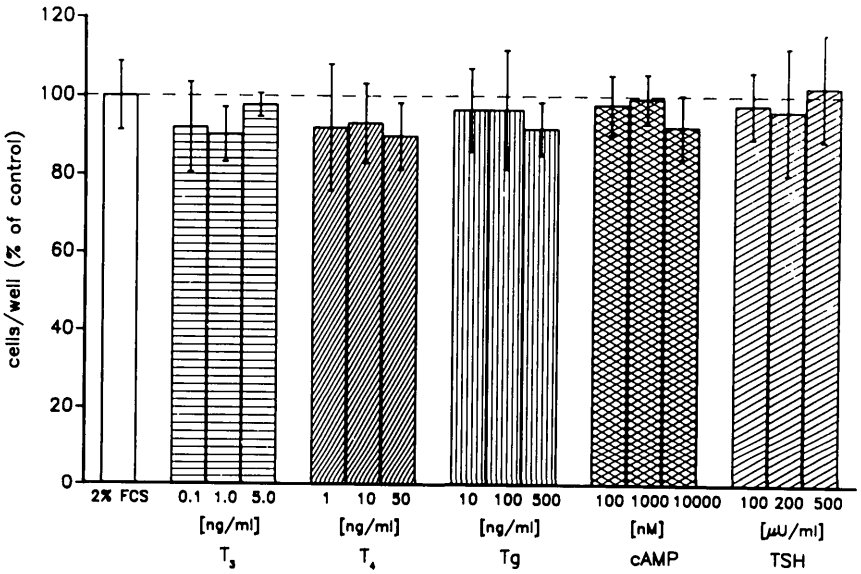

Fig. 6. Influence of T_3 (n = 4), T_4 (n = 6), Tg (n = 6), cAMP (n = 6), and TSH (n = 10) on endothelial cell growth at the indicated concentrations.

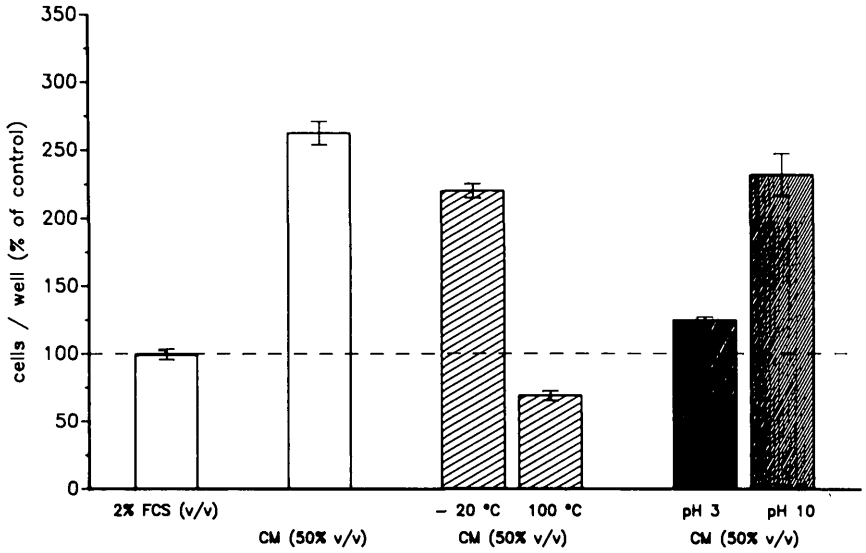

Fig. 7. Physicochemical Characterization of the Mitogenic Activity in CM
Endothelial cells were stimulated with CM (50%) (vol/vol) after freezing, boiling, alkaline, or acid treatment (n = 3).

follicles was significantly modified by treatment of the follicles with TSH, EGF, or by the addition of iodide, according to the direct effects of these substances on thyroid follicle cell proliferation (14). The mitogenic activity in CM from TSH-treated follicles on endothelial cells was significantly lower compared to CM from untreated or from EGF-treated thyroid follicles. These differences in mitogenic activity in CMs are not due to changes of cell numbers of thyroid follicles during the incubation time for conditioning the medium, since thyroid follicles were kept in suspension culture by the use of hydrophobic dishes. Under these conditions cell numbers were not influenced by EGF or TSH. These results indicate that addition of TSH and the resulting stimulation of specific functions in thyroid follicles either lowers the production or the release of the growth-stimulating activity or induces the production of an inhibitory activity. Since cultured thyroid follicles secrete thyroid hormones in response to TSH stimulation (13), it may be argued that these thyroid hormones are responsible for the observed decrease in the mitogenic activity in TSH-CM. This is unlikely since 1) T_3-CM or T_4-CM have no significant different mitogenic activity compared to basal CM, and 2) thyroid hormones have no effect on endothelial cell growth. Incubation of thyroid follicles with EGF significantly increased the growth-promoting

effect of CM on endothelial cells compared to both CM from unstimulated and TSH-treated follicles. As EGF does not influence endothelial cell growth and as EGF could not be detected in CM by RRA, it can be assumed that stimulation of thyroid follicles with EGF specifically enhances the mitogenic activity of CM on endothelial cells.

Addition of iodide during the incubation of thyroid follicles resulted in a suppression of the mitogenic activity in CM. Until now it is not clear, whether this suppression is due to the absence of the mitogenic factor(s) in CM or due to the presence of (an) additional factor(s), which may inhibit endothelial cell growth. Similar results with iodide-conditioned medium were obtained with fibroblasts (10, 11). The concentration of iodide, which was used, is completely organified by the follicles (13) and was found to inhibit the growth of thyroid follicles (14).

The modulation of the paracrine mitogenic activity in CM from isolated thyroid follicles on endothelial cells is comparable to the growth regulation of porcine thyroid follicles *in vitro*. TSH is not a growth factor for thyroid follicles, but even inhibits proliferation of thyroid cells (14). Similar results were found by others using isolated porcine thyroid follicles (30) or human thyroid cells (31, 32). EGF, IGF-I, or FCS-induced growth of thyroid follicles is inhibited by TSH and also by iodide (14, 33). This is in agreement with *in vivo* data which demonstrated that thyroid hyperplasia is not correlated with TSH serum levels during goiter induction, but inversely related to the iodine content of the thyroid gland (34). *In vitro* growth of thyrocytes stimulated by TSH has been demonstrated by several groups using primary monolayer cultures of thyrocytes (35–37) or thyroid follicles in suspension (38, 39). However, this was only found in the presence of insulin or FCS. Why insulin at low concentrations may act as a permissive factor as proposed by Smith *et al.* (38) for growth-stimulatory activity of TSH is yet not fully understood, perhaps by cross-reactivity with the IGF-I receptor; clearly in the absence of insulin no growth stimulatory effect of TSH was found (39). The FRTL 5 cell line can not be used for answering the question whether TSH is a growth factor for thyroid cells, since this cell line has been selected for the criterion to grow only in the presence of TSH (40). The different results obtained with thyroid monolayer cultures are most probably not due to different species used as donors of the thyroid cells, but more likely due to different culture conditions and to the loss of many specific cellular functions, which are preserved in cultures of thyroid follicles (13).

In summary, our results confirm the hypothesis that thyroid angiogenesis is due to, at least in part, one or more polypeptide factor(s) which are released by the epithelial thyroid cells and which may act in a paracrine manner on blood vessel cells. Further investigations will

Table 2. Effect of Trypsin Treatment on Mitogenic Activity in CM

	Cells/Well (% of Control)	
	No trypsin	Trypsin
CM	131.9 ± 16.4	104.1 ± 9.0
medium (MEM)	101.7 ± 1.3	100.2 ± 4.8

Endothelial cell growth was stimulated with CM or medium (30%) (vol/vol) after treatment with or without trypsin (n=3, mean ± SD).

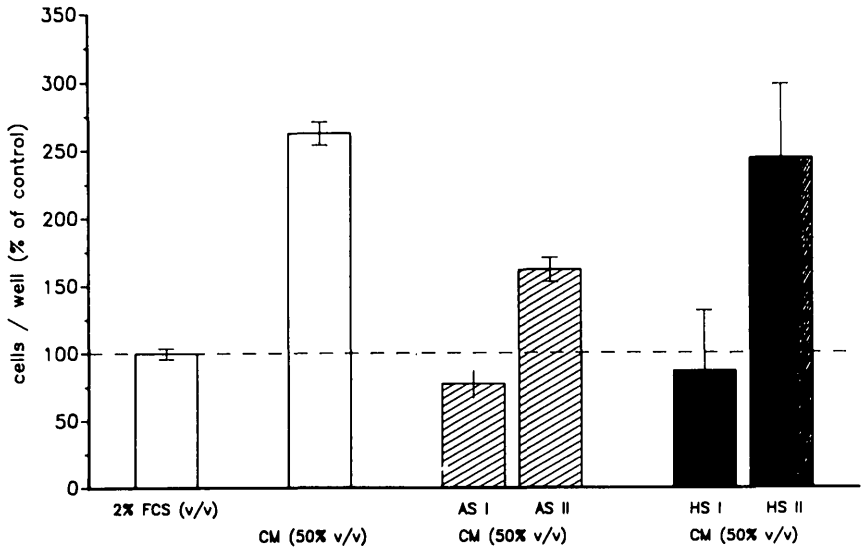

Fig. 8. Fractionation of the Mitogenic Activity in CM by Ammonium Sulfate Precipitation and Affinity Chromatography on Heparin-Sepharose

AS I: 1.9 M ammonium sulfate, AS II: 3.8 M ammonium sulfate, HS I: nonheparin-binding fraction, HS II: heparin-binding fraction (n = 3).

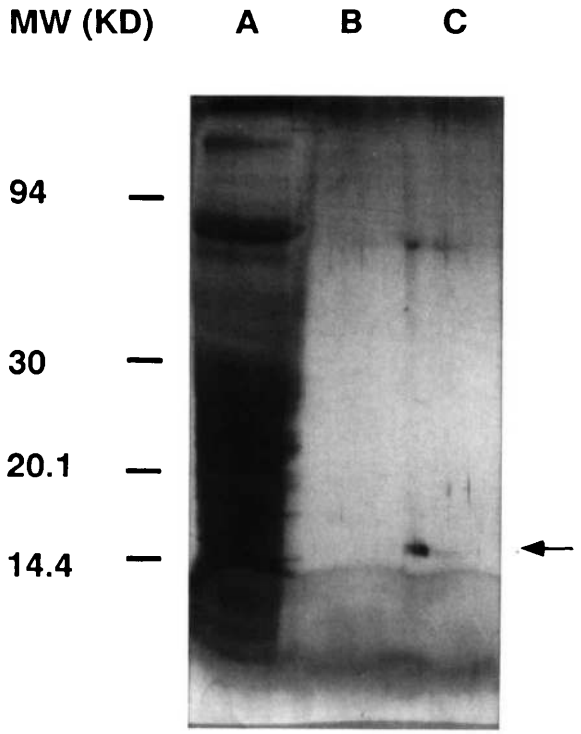

Fig. 9. SDS-Polyacrylamide Electrophoresis of CM Fractionated on Heparin Sepharose as described in *Materials and Methods*

Lane A: 0.6 M NaCl fraction; lane B: 1 M NaCl fraction; lane C: 2 M NaCl fraction. Twenty microliters of each sample were applied (=100-fold concentration *vs.* native CM). *Arrow*; Double band with a mol wt of 15 and 15.5 k. Mol wt standards were rabbit phosphorylase *b* (94 k), bovine carbonic anhydrase (30 k), soybean trypsin inhibitor (20.1 k), and bovine lactalbumin (14.4 k).

be necessary for more detailed information about the molecular structure of the paracrine factor(s).

MATERIALS AND METHODS

Materials

Collagenase (clostridium histolyticum), penicillin-streptomycin, transferrin, and trypsin (1:250) were obtained from Boehringer (Mannheim, FRG). FCS, heat-inactivated FCS, amphotericin B, and trypsin-EDTA (0.5%/0.2%) were from Gibco Europe (Karlsruhe, FRG). Minimal Essential Medium (MEM), Medium 199, soybean trypsin inhibitor, hydrocortisone, porcine skin gelatine, bovine TSH, mouse EGF, ECGS, and cAMP were obtained from Sigma Chemie (Deisenhofen, FRG). IGF-I and bovine bFGF was purchased from Amersham (Braunschweig, FRG). T_3 and T_4 were a gift from Henning (Berlin, FRG). Tg from porcine thyroid glands was prepared as described (41). All other reagents were from Merck (Darmstadt, FRG).

Hydrophobic Petriperm culture dishes (28 cm^2) were obtained from Heraeus (Hanau, FRG). Twenty four-mutiwell culture plates were from Costar/Tecnomara (Fernwald, FRG); plastic culture dishes (28 cm^2) and culture flasks (75 cm^2) were from Nunc (Wiesbaden, FRG).

RRA for EGF was obtained from Paesel (Frankfurt/M, FRG), and RIA for human IGF-I from Nichols Institute Diagnostics (San Juan Capistrano, CA). Cyclic AMP antibody was a gift from R. Gerzer, cAMP standards and tracer were from NEN (Dreieich, FRG). Heparin-sepharose CL-6B and trisacryl GF 05 were from LKB-Pharmacia (Freiburg, FRG).

Preparation of Isolated Thyroid Follicles

Thyroid follicles were isolated as recently described (13). In brief, thyroid glands were obtained from freshly slaughtered hogs. The surrounding tissue was removed and after infusion of collagenase soiution (1.0 mg/ml in MEM) into the blood vessels at the caudal pole the thyroid glands were incubated for 45 min in MEM at 37 C. The fibrous capsule was removed and isolated follicles could be released into MEM by gently shaking the digested tissues. After several washings in MEM closed or nearly intact thyroid follicles were obtained without contaminating single cells. The follicles were cultured in MEM supplemented with FCS (1%) (vol/vol), sodium hydrogen carbonate (2.2 g/liter), glucose (2 g/liter), penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (1 μg/ml) in Petriperm dishes supplied with a hydrophobic membrane (10^7 cells in 4 ml) for 24 h in a humidified atmosphere with 5% CO_2 at 37 C before further investigations.

Growth of Thyroid Follicles

After a resting phase of 24 h, thyroid follicles were washed twice in MEM and 10^5 cells per well were distributed into 24-well-culture-plates in 1 ml MEM containing transferrin (5 μg/ml), hydrocortisone (10 nM), and gelatine from porcine skin (10 mg/ml). The test substances were added in triplicate and the follicles were incubated for 6 days. For determination of cell numbers a cell suspension was obtained after incubation with PBS containing 25 mM EDTA, 40 mM Tris, pH 8.3 (750 μl per well) and trypsin (5 mg/ml)-EDTA (2 mg/ml) (250 μl/well) for 1 h and mechanical disruption of the follicles by vigorous pipetting. Cell numbers were determined with an adapted Coulter Counter.

Preparation of CM from Isolated Thyroid Follicles

Thyroid follicles corresponding to about 10^7 cells were kept in suspension in hydrophobic Petriperm dishes (28 cm^2) in 4 ml serum-free MEM containing trypsin-inhibitor (0.1 mg/ml), transferrin (5 μg/ml), and hydrocortisone (10 nM). Follicles were incubated either without any further additions for basal CM, with TSH (1 mU/ml) to produce TSH-CM, with EGF (5 ng/ml) for EGF-CM, with iodide (20 μM) for iodide-CM, with T_3 (1.5 ng/ml) for T_3-CM or with T_4 (50 ng/ml) for T_4-CM. After incubation for 72 h, the CM was obtained by centrifugation of the follicle suspension at 100 × *g* for 2 min and stored at 4 C. Determination of the cell numbers and viability of thyroid follicles after this 72-h incubation showed no significant difference between cells from basal-CM, TSH-CM, or EGF-CM; *e.g.* initial cell number before CM production was 1.53×10^7 cells per dish and after 72 h cell number from basal-CM was 1.49×10^7, from TSH-CM 1.46×10^7, and from EGF-CM 1.43×10^7 cells per dish. Viability was in the range of 90–95%.

Physicochemical Characterization of the Mitogenic Activity in CM

CM from thyroid follicles was desalted on a Tris-Acryl GF-05 column (2 × 20 cm) using ammonium acetate (50 mM) as elution buffer and concentrated by lyophilization. Samples were resolved in MEM, dialyzed against MEM for 24 h, sterilized by filtration, and substituted with antibiotics. For control, fresh medium was treated in the same manner.

Chemical stability of the mitogenic activity in CM was tested by freezing (−20 C for at least 24 h) and thawing, by boiling at 100 C for 10 min, by acid treatment with 1 M HCl to pH 3 or alkaline treatment with 1 M NaOH to pH 10 for 1 h followed

by dialysis against MEM for 24 h, centrifugation (10,000 × *g*, 2 min), sterile filtration and addition of antibiotics.

Treatment of CM (derived from incubations without trypsin inhibitor) with trypsin was performed with 10 μg/ml trypsin (170 benzylargininethylester units) for 60 min at 37 C; trypsin activity was stopped by the addition of trypsin inhibitor (150 μg/ml, ~10-fold excess). Controls were performed either with fresh medium by the same treatment or with CM without addition of trypsin.

Fractionated precipitation with ammonium sulfate was performed according to the method described by Gospodarowicz *et al.* (42). Equal volumes of CM and 3.8 M ammonium sulfate solution were mixed, incubated for 1 h, and centrifuged at 100,000 × *g* for 20 min. Ammonium sulfate was added to the supernatant in a final concentration of 3.8 M. The samples were then incubated again for 1 h and centrifuged at 100,000 × *g* for 20 min. Pellets were dissolved in distilled water and dialyzed with distilled water for 1 h and with MEM for 24 h. All steps were performed at 4 C. Finally, the samples were sterilized by filtration and antibiotics were added.

For chromatography on heparin-sepharose, up to 20 ml CM were applied to a heparin-sepharose CL-6B column (1 × 5 cm). The nonheparin-binding fraction was eluted with phosphate buffer (50 mM, pH 7.4) containing 0.15 M NaCl, the heparin-binding fraction was eluted with the same buffer containing 1.5 M NaCl. Fractions of each peak were pooled, concentrated 5-fold by dialysis against 25% polyethyleneglycol in PBS, pH 7.4, dialyzed against PBS, pH 7.4, for 24 h and for additional 24 h against MEM. Finally, after centrifugation (10,000 × *g*, 2 min), the samples were sterilized by filtration and antibiotics were added.

For gel electrophoretic analysis, CM was fractionated on heparin-sepharose with 0.6, 1, and 2 M NaCl according to Gospodarowicz *et al.* (43); samples were concentrated after dialysis by lyophilization, dissolved and boiled in electrophoresis sample buffer containing 1% SDS, 5% mercaptoethanol, and were run on a SDS-polyacrylamide slab gel (44) with 10% and 4% acrylamide in the running and stacking gels, respectively. Staining of the proteins was performed by a silver staining method as described (45).

Determination of IGF-I and EGF in CM

Control-CM was used for determination of IGF-I and EGF. IGF-I was measured after acidic extraction according to Clemmons *et al.* (46) with a commercial RIA kit; the lower limit of detection was 0.5 ng/ml. EGF was determined by a commercial RRA or by a RRA using a crude membrane preparation from porcine thyroid glands as described (47); the lower limit of detection was 1 ng/ml.

Preparation and Culture of Endothelial Cells

Endothelial cells were obtained from aortae of freshly slaughtered adult hogs according to the method of Jaffe (48). Briefly, porcine aortae (20–25 cm long) were filled with collagenase solution (1.2 mg/ml in PBS) and digested for 15 min at 37 C. The cell suspensions were harvested and the vessels were washed with PBS; cell suspensions and washing solutions were combined and medium 199 with 10% FCS was added. The cells were washed four times with medium containing 10% FCS and after testing cell viability by staining with ethidium bromide and acridine orange, the cell number was determined in a Neubauer chamber. Usually cell viability was about 97% and the average yield of isolated endothelial cells was 1.65×10^6 cells per vessel (n = 82).

Cells were cultured in Medium 199, generally supplied with soybean trypsin-inhibitor (0.1 mg/ml), sodium hydrogen carbonate (2.2 g/liter), glucose (2 g/liter), penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (1 μg/ml), and heat-inactivated FCS (10%) on 75 cm^2 culture flasks coated with porcine skin gelatine. Culture conditions were 5% CO_2 at 37 C in a humidified atmosphere. The culture medium was changed 24 h after preparation and from that time on every 72 h. When cells were grown to confluency after approximately 6 days, splitting was performed using collagenase solution (1.2 mg/ml in PBS). Cell cultures were used until day 20 after the preparation to avoid cellular dedifferentiation and overgrowth by contaminating fibroblasts and smooth muscle cells. Confluent monolayers of endothelial cells exhibited the typical cobblestone growth pattern with polygonal cells. Endothelial cells were characterized by their ability to produce angiotensin converting enzyme (ACE) and by their specific prostaglandin pattern. Culture medium had a total ACE activity of 642.0 ± 338.0 pmol/10^6 cells × min; cell lysates had only 117.0 ± 29.3 pmol/10^6 cells × min, as measured by a fluorimetric assay (49). The enzyme activity could be inhibited by the specific ACE blocker MK 422. PG synthesis was studied after labeling of the cells with ^{14}C-arachidonic acid; three peaks corresponding to PGE_2, $PGF_{2\alpha}$, and 6-keto-$PGF_{1\alpha}$ were found in cell lysates and supernatants by TLC.

Growth Experiments with Endothelial Cells

Endothelial cells were seeded into gelatine-coated 24-well plates at an initial cell number of 10,000 cells per well and cultured in medium 199 containing 10% FCS for 24 h, followed by a 24-h period with a serum reduction to 1% FCS. Thereafter, cell growth was initiated with CM or growth factors in the presence of 2% FCS in medium 199. After 72-h incubation time the cells were harvested with trypsin-EDTA solution in PBS (0.5 mg/ml trypsin, 0.2 mg/ml EDTA), and cell numbers were determined using an adapted Coulter Counter. With 2% FCS as control value, the initial cell number of 10,000 cells per well had increased 5-fold after 5 days (mean 51,000 cells per well from seven different experiments); since a considerable variation between separate cell preparations was found (range, from 45,000–69,000 cells per well); the results obtained after stimulation with CM or growth factors were related to the individual control values (2% FCS = 100%).

Determination of T_3, T_4, Tg, and cAMP

Concentrations of T_3, T_4, Tg, and cAMP were determined by RIA as described (13, 50, 51).

Data Presentation and Statistical Analysis

All experiments were performed in duplicate or triplicate. Pooled data from separate experiments are presented as means ± SD. A multivariant analysis of the data was performed (ANOVA), and significant differences were evaluated by the Bonferroni test. Using ANOVA to prove whether data could be pooled, we found a highly significant effect for different conditioned media (basal CM, TSH-CM, EGF-CM) ($P < 0.005$). The influence of separate endothelial cell preparations was also significant, but on a much lower level ($P < 0.05$); this influence was considered as only a minor effect and allowed us therefore to pool data from separate experiments. Within one experiment no significant effect was found for duplicate or triplicate determinations.

Acknowledgments

We are indepted to Prof. Dr. H. Dahlheim and his co-worker Dipl. Biol. W. Reiter (Physiologisches Institut der Universität München) for determination of angiotensin converting enzyme activities; to Dr. B. Olgemöller (Klinisch-chemisches Institut und Forschergruppe Diabetes, Städt. Krankenhaus München-Schwabing) for his help in establishing endothelial cell cultures. We thank Dr. A. König for his help in statistical analysis and Dr. R. Gerzer for supplying us with cAMP antibody (both Medizinische Klinik Innenstadt der Universität München). We gratefully acknowledge Mrs. R. Buric, Mrs. A, Isemann and Ms. P. Rank for excellent technical assistance.

Received July 18, 1988. Revision received February 3, 1989. Accepted February 13, 1989.

Address requests for reprints to: Dr. W. Greil, Medizinische Klinik Innenstadt der Universität München, Ziemssenstraße 1, D-8000 München 2, Federal Republic of Germany

Supported by the Deutsche Forschungsgemeinschaft.

REFERENCES

1. James R 1984 Polypeptide growth factors. Annu Rev Biochem 53:259–292
2. Franchimont P 1986 Clinics in endocrinology and metabolism. Paracrine Control (ed). Saunders Co, London, vol 15:1
3. Frederick JL, Shimanuki T, DiZerega GS 1984 Initiation of angiogenesis by human follicular fluid. Science 224: 389–390
4. DiZerega GS, Marut EL, Turner CK, Hodgen GD 1980 Asymmetrical ovarian function during recruitment and selection of the dominant follicle in the menstrual cycle of the rhesus monkey. J Clin Endocrin Metab 51:698–701
5. Gospodarowicz D, Thakral KK 1978 Production of a corpus luteum angiogenic factor responsible for proliferation of capillaries and neovascularization of the corpus luteum. Cell Biol 75:847–852
6. Folkman J 1985 Tumor angiogenesis. Adv Cancer Res 43:175–203
7. Wollman SH, Herveg JP, Zeligs JD, Ericson LE 1978 Blood capillary enlargement during the development of thyroid hyperplasia in the rat. Endocrinology 102:2306–2314
8. Smeds S, Wollman SH 1983 ^{3}H-Thymidine labeling of endothelial cells in thyroid arteries, veins and lymphatics during thyroid stimulation. Lab Invest 48:285–291
9. Many MC, Denef JF, Haumont S 1984 Precocity of the endothelial proliferation during a course of rapid goitergenesis. Acta Endocrinol (Copenh) 105:487–491
10. Greil W, Gärtner R, Rettenmaier U, Horn K, Pickardt C 1986 Implication for a paracrine factor from porcine thyroid follicles with growth promoting activity for fibroblasts. In: Medeiro-Neto G, Gaitan E (eds) Frontiers in Thyroidology. Plenum Medical Book Company, New York and London, vol 2:941–943
11. Greil W, Gärtner R, Rettenmaier U, Horn K, Pickard C 1986 Secretion of a paracrine factor with growth promoting activity for fibroblasts from porcine thyroid follicles. Acta Endocrinol [Suppl] (Copenh) 274:45 (Abstract)
12. Gärtner R, Bechtner G, Stübner D, Greil W 1987 Paracrine interaction between thyrocytes and fibroblasts. Acta Endocrinol [Suppl] (Copenh) 281:225–230
13. Gärtner R, Greil W, Stübner D, Permanetter W, Horn K, Pickardt CR 1985 Preparation of porcine thyroid follicles with preserved polarity: functional and morphological properties in comparison to inside-out follicles. Mol Cell Endocrinol 40:9–16
14. Gärtner R, Greil W, Demharter R, Horn K 1985 Involvement of cyclic AMP, iodide and metabolites of arachidonic acid in the regulation of cell proliferation of isolated porcine thyroid follicles. Mol Cell Endocrinol 42:145–155
15. Hayes LW, Goguen ChA, Cing SF, Slakey LL 1978 Angiotensin-converting enzyme: accumulation in medium from cultured endothelial cells. Biochem Biophys Res Comm 82:1147–1153
16. Ody C, Seillan C, Russo-Marie F 1982 6-ketoprostaglandin $F_{1\alpha}$, prostaglandins E_2, $F_{2\alpha}$ and thromboxane B_2 production by endothelial cells, smooth muscle cells and fibroblasts cultured from piglet aorta. Biochim Biophys Acta 712:103–110
17. Thomas KA, Gimenez-Gallego G 1986 Fibroblast growth factors: broad spectrum mitogens with potent angiogenic activity. Trends Biochem Sci 11:81–84
18. Gospodarowicz D, Neufeld G, Schweigerer L 1986 Fibroblast growth factor. Mol Cell Endocrinol 46:187–204
19. Gospodarowicz D, Ferrara N, Schweigerer L, Neufeld G 1987 Structural characterization and biological functions of fibroblast growth factor. Endocr Rev 8:95–114
20. Stout W 1983 Hormone-endothelial interactions. In: Cryer A (ed) Biochemical Interactions at the Endothelium. Elsevier Science Publishers, pp 301–312
21. Gospodarowicz D, Greenburg G, Bialecki H, Zetter BR 1978 Factors involved in the modulation of cell proliferation in vivo and in vitro: the role of fibroblast and epidermal growth factors in the proliferative response of mammalian cells. In Vitro 14:85–118
22. Miller MJ, Fels EC, Shapiro LE, Surks MI 1987 L-Triiodothyronine stimulates growth by means of an autocrine factor in a cultured growth hormone-producing cell-line. J Clin Invest 79:1773–1781
23. Lobb RR, Harper JW, Fett JW 1986 Purification of heparin-binding growth factors. Anal Biochem 154:1–14
24. Gospodarowicz D, Cheng J, Lui GM, Baird A, Esch F, Bohlen P 1985 Corpus luteum angiogenic factor is related to fibroblast growth factor. Endocrinology 117:2383–2391
25. Ueno N, Baird A, Esch F, Ling N, Guillemin R 1987 Isolation and partial characterization of fibroblast growth factor from bovine testes. Mol Cell Endocrinol 49:189–194
26. Schweigerer L, Neufeld G, Friedman J, Abraham JA, Fiddes JC, Gospodarowicz D 1987 Basic fibroblast growth factor: production and growth stimulation in cultured adrenal cortex cells. Endocrinology 120:796–800
27. Goodmann AL, Ronn JD 1987 Thyroid angiogenesis: endotheliotropic chemoattractant activity from rat thyroid cells in culture. Endocrinology 121:2131–2140
28. Mak WW, Bachrach LK, Eggo MC, Burrow GN Growth promoting activity and plasminogen activator activity in ovine thyroid cells in primary culture. 7th International Congress of Endocrinology, Quebec City, Quebec, Canada, 1984, p 1081 (Abstract)
29. Mak WW, Bhaumick B, Bala RM, Kudlow JE, Eggo MC, Burrow GN Detection of insulin-like growth factors in the conditioned media of primary cultures of ovine thyroid cells. American Society of Clinical Investigators, Washington, DC, 1985, vol 33:536A (Abstract)
30. Watanabe Y, Amino N, Tamaki H, Iwatani Y, Miyani K 1985 Bovine thyrotropin inhibits DNA synthesis inversely with cyclic AMP production in cultured porcine thyroid follicles. Endocrinol Jpn 32:81–88
31. Westermark B, Karlsson FA, Wålinder O 1979 Thyrotropin is not a growth factor for human thyroid cells. Proc Natl Acad Sci USA 76:2022–2026
32. Errick JE, Ing KWA, Eggo MC, Burrow GN 1986 Growth and differentiation in cultured human thyroid cells: effects of epidermal growth factor and thyrotropin. In vitro 22:28–36
33. Gärtner R, Greil W 1986 The mitotic activity of IGFI, insulin and EGF on isolated porcine thyroid follicles is under negative control of TSH and cAMP. Ann Endocrinol p 66 (Abstract)
34. Stübner D, Gärtner R, Greil W, Gropper K, Brabant G, Permanetter W, Horn K, Pickardt CR 1987 Hypertrophy and hyperplasia during goiter growth and involution in rats—separate bioeffects of TSH and iodine. Acta Endocrinol (Copenh) 116:537–548
35. Dumont JE, Roger P, van Henverswyn B, Erneux C, Vassart G 1984 Control of growth and differentiation by known intracellular signal molecules in endocrine tissues: the example of the thyroid gland. Adv Cycl Nucl Prot Phosph Res 17:337–342
36. Dere WH, Rapoport B 1986 Control of growth in cultured rat thyroid cells. Mol Cell Endocrinol 44:195–199
37. Roger PP, Servais P, Dumont JE 1983 Stimulation by thyrotropin and cyclic AMP of the proliferation of quiescent

canine thyroid cell cultures in a defined medium containing insulin. FEBS Lett 157:323–329
38. Smith P, Williams ED, Wynford-Thomas D 1987 *In vitro* demonstration of a TSH-specific growth desensitising mechanism in rat thyroid epithelium. Mol Cell Endocrinol 51:51–58
39. Williams DW, Wynford-Thomas D, Williams ED 1987 Control of human thyroid follicular cell proliferation in suspension and monolayer culture. Mol Cell Endocrinol 51:33–40
40. Ambesi-Impiombato FS, Parks LAM, Coon HG 1980 Culture of hormone-dependent epithelial cells from rat thyroids. Proc Natl Acad Sci USA 77:3455–3459
41. Gärtner R, Bechtner G, Greil W, Horn K, Pickardt CR 1985 Characterization of microheterogeneity of human thyroglobulin from different thyroid disorders. Acta Endocrinol (Copenh) 109:76–82
42. Gospodarowicz D, Bialecki H, Greenburg G 1978 Purification of the fibroblast growth factor activity from bovine brain. J Biol Chem 253:3736–3743
43. Gospodarowicz D, Baird A, Cheng J, Lui GM, Esch F, Bohlen P 1986 Isolation of fibroblast growth factor from bovine adrenal gland: Physiochemical and biological characterization. Endocrinology 118:82–90
44. Lämmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227:680–685
45. Merril CR, Goldman D, Sedman SA, Ebert MH 1981 Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Nature 211:1437–1438
46. Clemmons DR, Underwood LE, Van Wyk JJ 1981 Hormonal control of immunoreactive somatomedin production by cultured human fibroblasts. J Clin Invest 67:10–19
47. Gärtner R, Tzavella C, Bechtner G, Greil W 1987 Evidence that thyroid growth promoting activity of immunoglobulin preparations is due to contamination with EGF. Acta Endocrinol [Suppl] (Copenh) 281:256–259
48. Jaffe EA 1984 Culture and identification of large vessel endothelial cells. In: Jaffe EA (ed) Biology of Endothelial Cells. Martinus Nijhoff Publishers, Boston, MA, pp 1–13
49. Rosenthal J, Rothemund J, Lutterotti Nv, Thrunreiter M, Reiter W, Kazda S, Garthoff B, Jakob I, Dahlheim H 1987 Effect of hypotensive agents on the renin-angiotensin system in vascular walls of spontaneously hypertensive rats. J Hypertension 5 [Suppl 2]:59–66
50. Gärtner R, Kewenig M, Horn K, Scriba PC 1980 A new principle of thyroxine (T_4) and triiodothyronine (T_3) radioimmunoassay in unextracted serum using antisera with binding optima at extreme pH ranges. J Clin Chem Clin Biochem 18:571–577
51. Gärtner R, Horn K, Pickardt CR 1980 Improvement of the diagnostic validity of the thyroglobulin radioimmunoassay. Acta Endocrinol [Suppl] (Copenh) 234:30–31 (Abstract)