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Tie-2 Is Expressed on Thyroid Follicular Cells, Is Increased in Goiter, and Is Regulated by Thyrotropin through Cyclic Adenosine 3′,5′-Monophosphate*

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ABSTRACT

Angiogenesis is coordinated with follicular cell growth in goitrogenesis. The angiopoietins, Ang-1 and Ang-2, are angiogenic growth factors acting through Tie-2, a tyrosine kinase receptor. We have examined the expression and regulation of the angiopoietins and Tie-2 in human and rat thyroids. In human goiters there was increased Tie-2 immunostaining, compared with that in normal thyroids, on both follicular and endothelial cells. In an induced goiter in rats, *in situ* hybridization showed increased expression of messenger ribonucleic acids (mRNAs) for Tie-2 and Ang-1 in follicular cells. As Tie-2 has previously been believed to be restricted to cells of endothelial lineage in adults, we examined its expression further in isolated follicular cells. Tie-2 and Ang-1 mRNA expression in human thyrocytes was confirmed by ribonuclease protection assay. Ang-2 mRNA was not detected in human cultures or rat thyroids. In both human follicular cell cultures and FRTL-5 cells, immunoblotting showed that Tie-2 expression was increased by TSH and agents that increased intracellular cAMP. In conclusion, we have demonstrated the expression of Tie-2 and Ang-1 in thyroid epithelial and endothelial cells, and have shown the regulation of Tie-2 by TSH and cAMP in follicular cells. Tie-2 expression is increased in goiter in both humans and rats, consistent with a role in goitrogenesis. (*J Clin Endocrinol Metab* **86:** 2709–2716, 2001)

PALPABLE GOITER OF diverse etiology affects up to 15% of the population, and its incidence may be higher in areas of iodine deficiency (1). The thyroid is a highly vascularized organ with a resting blood flow of 5 mL/g·min that can increase greatly in disease, including goiter. Goiter is thus an excellent model for physiological angiogenesis and for investigating the regulation of angiogenic growth factors. In experimental models of induced goiter both [^{3}H]thymidine labeling of endothelial cells and capillary diameter increase, and this occurs within a few days of goitrogen administration (2). Endothelial cell proliferation precedes that of follicular cells, consistent with the hypothesis that neovascularization is a prerequisite for thyroid growth (3, 4). Several angiogenic factors are synthesized by thyroid follicular cells, suggesting that the stimulus for endothelial growth may come from paracrine secretion from follicular cells in response to TSH and other goitrogenic factors. These include fibroblast growth factors (FGF-1 and FGF-2) (5), vascular endothelial growth factor (VEGF), and placental growth factor (6). Conversely the antiangiogenic factor thrombospondin reduces to negligible levels within 2 weeks of goitrogen treatment in rats (7).

The angiopoietins are a recently discovered family of angiogenic growth factors whose receptor, Tie-2, is reported to be confined to the endothelium (8–10) and to hemopoietic cells in adults (11). Gene knockout studies in mice have shown an essential role for Tie-2 and the angiopoietins in blood vessel development. Disruption of Tie-2 resulted in embryonic lethality on day 10.5, with defects in vascular remodeling (9). Tie-2 is a membrane tyrosine kinase receptor for which there are two known ligands, angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2). Ang-1 is the agonist of Tie-2 and initiates autophosphorylation of Tie-2 on tyrosine residues, which promotes capillary sprouting and blood vessel maturation (12, 13). Mouse models have shown that overexpression of Ang-1 increases capillary density and vascular branching, whereas targeted gene knockout of Ang-1 causes fetal death with characteristic vascular defects (14). Overexpression of Ang-2 in transgenic mice is lethal and appears to cause the same vascular defects as knockout of Ang-1 (15). This is consistent with an *in vivo* role of Ang-2 as an antagonist of Ang-1, and in endothelial cells, Ang-2 has been shown to bind to Tie-2 without causing autophosphorylation. The overall effect of Ang-2 in adults is to destabilize blood vessels, which may result in vessel regression if Ang-2 is acting alone or robust angiogenesis if it is expressed with another proangiogenic factor such as VEGF (16–18). These growth factors and their receptor, Tie-2, are important in the cyclical angiogenesis associated with the female reproductive cycle (17), in placental development (19), and in tumor

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growth (18, 20–22), and there is intense interest in the control of their synthesis.

The aim of this study was to examine the expression and regulation of the angiopoietins and their receptor Tie-2 in the thyroid. We show that Tie-2 receptor protein is expressed in normal human thyroid glands and increased in multinodular goiter. Surprisingly, we also found that Tie-2 was present on follicular cells as well as endothelial cells. To investigate this novel observation further we used primary cultures of functional human follicular cells and the clonal rat thyroid cell line, FRTL-5, to examine the regulation of Tie-2, Ang-1, and Ang-2 expression by TSH and cAMP, which mediates most, if not all, of the effects of TSH. To confirm these *in vitro* findings, we have also studied the *in vivo* expression of the messenger ribonucleic acids (mRNAs) for the angiopoietins and Tie-2 in goiter development in rats using *in situ* hybridization.

Materials and Methods

Immunohistochemistry

Human surgical specimens were fixed in 10% formaldehyde and embedded in paraffin wax after serial dehydration through 70%, 90%, and 100% ethanol and xylene. Sections of 5 μm were cut and mounted on glass slides. Sections were rehydrated through xylene and ethanol. Endogenous peroxidase was blocked by incubation with 4% H_2O_2 in methanol for 10 min. The sections were washed in PBS (10 mmol/L Na_2HPO_4, 2.7 mmol/L KCl, and 137 mmol/L NaCl, pH 7.4) twice and incubated in 10% normal goat serum in PBS to block nonspecific protein binding. Rabbit polyclonal antibody raised to an epitope at the carboxyl-terminus of Tie-2 (sc-320, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was diluted in 10% normal goat serum in PBS and incubated with the sections in a humidified chamber for 1 h at room temperature. After two washes with PBS, secondary biotinylated goat antirabbit antibody was applied for 30 min. Following two washes with PBS, the sections were incubated with ExtrAvidin-Peroxidase (Sigma, St. Louis, MO) for 30 min. Antigen immunoreactivity was localized using freshly prepared 3,3′-diaminobenzidine [1.89 mmol/L containing 0.03% of 30% (vol/vol) H_2O_2 (Sigma, Poole, UK)]. The slides were washed in PBS, counterstained with Meyer's hemalum, dehydrated in ascending ethanol, cleared with xylene, mounted under glass coverslips with DPX mountant (Raymond A Lamb, London, UK), and assessed by light microscopy. Primary antibody was omitted in negative controls. Preabsorption of antibody with immunizing antigen (sc-320p, Santa Cruz Biotechnology, Inc.) was also performed. Positive controls of ovary were used (17).

Cell culture

Human thyroid follicular cells were prepared from surgical specimens as previously described (23). In brief, thyroid tissue (normal and multinodular goiter) was digested using 0.2% collagenase. Follicles were plated in the medium described by Ambesi-Impiombato *et al.* (24) supplemented with TSH (300 mU/L), insulin (100 μg/L), penicillin (10^5 U/L), streptomycin (100 mg/L), and 1% newborn bovine calf serum. After 72 h serum was omitted. The cells were grown for 7 days before experimentation, and the medium was changed every 3 days. On day 7 the medium was changed, and TSH was omitted for 72 h. The cells were incubated for 3 days in TSH, forskolin, or dibutyryl cAMP in various concentrations, and culture was terminated by removal of medium and lysis of the cells with 1% SDS in 62.5 mmol/L Tris-HCl, pH 6.8.

The rat thyroid cell line FRTL-5 was a gift from Dr. L. D. Kohn (Interthyr Foundation, Bethesda, MD). Cells were grown in the same medium supplemented with TSH (300 mU/L), insulin (1 mg/L), penicillin (10^5 U/L), streptomycin (100 mg/L), and 5% newborn bovine calf serum. TSH was omitted for 7 days before experimentation with TSH, forskolin, or 8-bromo-cAMP, and the cells were lysed as described above.

Western blots

Proteins in 1% SDS were heated at 100 C for 10 min. The protein concentration was assayed using a protein assay (Bio-Rad Laboratories, Inc., Hercules, CA). One hundred micrograms of protein were reduced by boiling in 2% 2-mercaptoethanol. Proteins were analyzed by electrophoresis on denaturing 10% SDS-PAGE with 5% stacking gel. The protein was transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). Membranes were blocked in 10% milk in TBST (10 mmol/L Tris, 100 mmol/L NaCl, and 0.1% Tween-20, pH 7.5) for 1 h and incubated in primary antibody at optimal dilution in 5% milk in TBST. The membrane was washed three times for 15 min each time in TBST and placed in antirabbit Ig conjugated to horseradish peroxidase secondary antibody (Santa Cruz Biotechnology, Inc.) for 1 h. Membranes were developed using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Aylesbury, UK) and exposed to x-ray film (Biomax MR, Eastman Kodak Co., Rochester, NY) with intensifying screens. Membranes were stripped with 62.5 mmol/L Tris, 2% SDS, and 0.75% 2-mercaptoethanol, pH 6.8, at 60 C for 30 min before reprobing. Antibody was incubated with blocking peptide in a 5-fold weight excess in blocking experiments. Antibodies used were C-term Tie-2 (sc320, Santa Cruz Biotechnology, Inc.) and its blocking peptide (sc320p, Santa Cruz Biotechnology, Inc.), N-term Tie-2 (sc9026, Santa Cruz Biotechnology, Inc.), and cytokeratin-8 (The Binding Site, Birmingham, UK). Densitometry was performed on UVP gelbase image analysis software (UVP, Cambridge, UK).

Rat goiter

Goiter in rats was induced by a low iodine (0.05 parts iodine/million) and methimazole (0.01%, wt/vol) diet as previously described (7).

In situ hybridization

The human Ang-1 probe consisted of a cDNA fragment in the vector pBluescript KS^+ (Stratagene), corresponding to nucleotides 240–804 of the sequence described by Davis (25). Digoxigenin-labeled antisense riboprobes for Ang-1 were transcribed by T3 RNA polymerase after linearization with *Not*I. Sense riboprobes for Ang-1 were transcribed by T7 RNA polymerase after linearization with *Xho*I.

The Ang-2 probe template consisted of nucleotides 1–635 of the sequence described by Maisonpierre (15) in the vector pBluescript KS^+. Antisense riboprobes were transcribed by T3 RNA polymerase after linearization with *Eco*RI. Sense riboprobes for Ang-2 were transcribed by T7 RNA polymerase after linearization with *Xho*I.

The Tie-2 probe template consisted of nucleotides 355-1386 of the sequence described by Ziegler *et al.* (26), also in pBluescript KS^+ vector. Antisense riboprobes were transcribed by T3 RNA polymerase after linearization with *Not*I. Sense riboprobes for Tie-2 were transcribed by T7 RNA polymerase after linearization with *Xho*I. All probes were transcribed in the presence of digoxigenin-11-UTP using the commercially available RNA color kit (Amersham Pharmacia Biotech).

In situ hybridization was carried out as previously described (27). Pretreated sections were hybridized in hybridization buffer containing either digoxigenin-labeled sense or antisense riboprobe. Hybridization was carried out in a humidified oven at 55 C overnight. After sequential washing in TBS buffer (10 mmol/L Tris and 100 mmol/L NaCl), the sections were incubated with a sheep antidigoxigenin antibody for 3 h. The sections were washed, and the signal was developed by incubation in a solution containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate at 4 C for 24–48 h. After development, sections were washed in distilled water and mounted.

Ribonuclease (RNase) protection assays

Total RNA was isolated from thyroid follicular cell cultures using TRIzol as described in the manufacturer's protocol (Life Technologies, Inc., Paisley, Scotland). The human Ang-1 probe template was described above. Ang-1 antisense RNA was transcribed by T3 RNA polymerase after linearization with *Not*I in the presence of [^{32}P]UTP (Amersham Pharmacia Biotech) using standard methods and on hybridization-protected 570 nucleotides. The Ang-2 probe template was constructed as described above, except that antisense RNA was transcribed by T3 RNA

polymerase after linearization with *Nco*I and on hybridization protected 388 nucleotides. The Tie-2 receptor probe protected 265 nucleotides after hybridization and was transcribed from the same construct as that used for *in situ* hybridization after linearization with *Sty*I. A probe for 28S ribosomal RNA was used as an internal standard and was transcribed from the pTRI-RNA-28S antisense control templates (Ambion, Inc., Witney, UK) with T3 RNA polymerase. On hybridization this probe protected 115 nucleotides.

Probes for Ang-1, Ang-2, Tie-2 ($1.0–5.0 \times 10^5$ cpm), and 28S ribosomal RNA ($1.0–5.0 \times 10^4$ cpm) were combined with 10 μg total RNA and coprecipitated. RNase protection assays were then performed using the RPA II kit (Ambion, Inc.) according to the manufacturer's instructions. Protected fragments were resolved by electrophoresis on 6% denaturing PAGE gels and dried onto Whatman 3MM paper (Clifton, NJ) under vacuum in a gel drier. Yeast RNA controls were run as well as uncut probe on each gel. Autoradiography was performed using x-ray film (Kodak Biomax MR) with intensifying screens at −70 C for 2–3 days.

Statistical analysis

The intensity of staining for Tie-2 in goiter specimens compared with that in controls was analyzed by Welch's *t* test. Western blots were performed a minimum of three times. Changes in the ratios of the densitometry reading/control reading were analyzed by ANOVA using Student-Newman-Keuls posttest, and the mean ± SEM are shown.

Results

Tie-2 is expressed on human follicular cells and increased in goiter

To determine whether human follicular cells expressed Tie-2, sections from normal human thyroid were stained with a polyclonal antibody raised against the carboxyl-terminus of Tie-2 (Santa Cruz Biotechnology, Inc.). There was specific staining of the endothelial cells (Fig. 1A). In sections from multinodular goiters, there was staining for Tie-2 on the endothelial cells and also on the follicular cells (Fig. 1B). Figure 1C shows a higher power magnification of Tie-2 staining in a follicle from a section of multinodular goiter where follicular cell staining is clearly visible. The increased follicular staining was not uniform across the thyroid sections, and there were areas with increased intensity and areas where there was relatively little staining. There was no positive staining of colloid within the follicles or on fibroblasts, lymphocytes, parafollicular cells, or stroma. Endothelial cells stained with different intensities depending on the size of the blood vessel, with small capillaries having a higher intensity than arterioles.

We compared staining in 15 multinodular goiter thyroids with that in 4 normal thyroids, and the results are shown in Fig. 2. The intensity of the staining of each follicle was recorded on a scale of 0–4 (0 = no staining, 4 = intense staining). For each thyroid section a line was drawn at random through the section, and each follicle that touched the line was scored. Blood vessels were excluded from the analysis. The total score for the line was calculated and divided by the number of follicles to give an intensity score per follicle. This was repeated for three lines by two independent observers for each thyroid. The score for the normal thyroids was 0.96 (SD = 0.28), and that for the multinodular goiters was 2.59 (SD = 1.167). The difference was significant ($P < 0.05$, using Welch's *t* test).

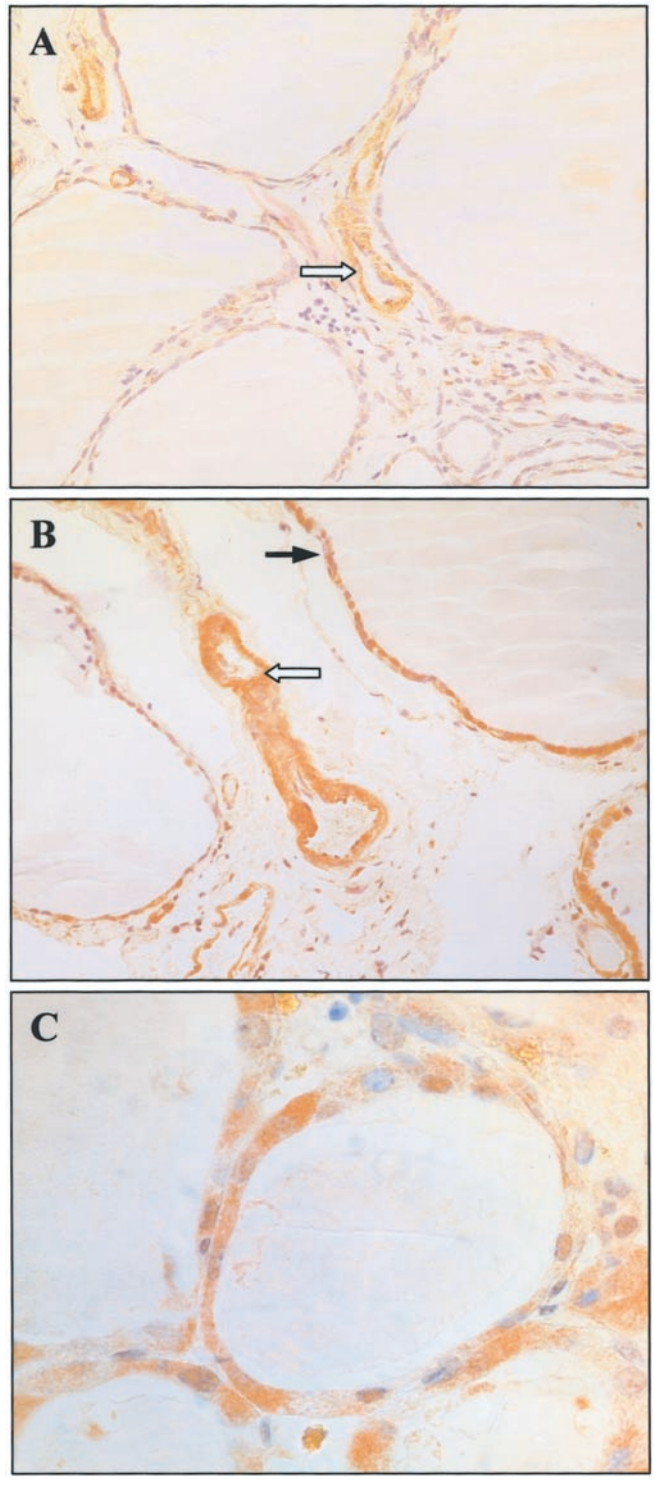

FIG. 1. Immunohistochemistry of human paraffin-embedded 5-μm sections of thyroid gland incubated with C-term Tie-2 antibody, and horseradish peroxidase-conjugated secondary antibody and stained with 3,3′-diaminobenzidine. A, Normal thyroid stained for Tie-2, with positive staining over the endothelial cells of a large perifollicular capillary (*white arrow*; magnification, ×400). B, Representative area of multinodular goiter showing specific intense staining over follicular cells (*black arrow*), with a comparable intensity to the staining over the endothelial cells (*white arrow*; magnification, ×400). C, High magnification of a follicle in multinodular goiter with staining over the follicular cells (magnification, ×1000).

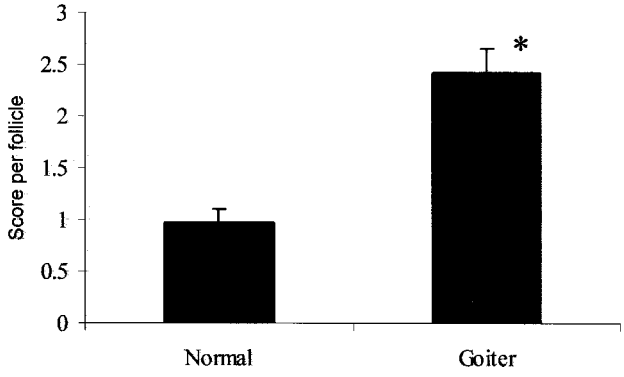

FIG. 2. Intensity of staining per follicle comparing 15 multinodular goiters with 4 normal thyroids. The average score per follicle with SE is shown. *, $P < 0.05$ *vs.* normal.

Tie-2 and Ang-1 mRNA are expressed in rat goiter

Goiter was induced in Fischer rats for 1 week as previously described (7). Sections from three control rat thyroids and three goitrogen-treated thyroids were examined by *in situ* hybridization for Ang-1, Ang-2, and Tie-2. The untreated control rats expressed no Ang-1, Ang-2, or Tie-2 mRNA on *in situ* hybridization using antisense probes (data not shown). After 1 week of goiter induction there was positive staining for both Tie-2 and Ang-1 (Fig. 3, A and B, respectively). Control sections using sense probes to Tie-2 and Ang-1 showed no staining (Fig. 3, C and D, respectively). The staining was localized to areas within the gland that stained very strongly, surrounded by areas with little or no staining, in a similar pattern to the Tie-2 immunostaining in human thyroid specimens. The *in situ* staining for both Tie-2 and Ang-1 was not restricted to the endothelial cells and predominantly appeared within follicular cells in the hyperplastic goiter (Fig. 3, E and F, respectively). There was no Ang-2 demonstrable by *in situ* hybridization in the goiter.

Tie-2 is present on thyroid follicular cells and is regulated by TSH

The total cell proteins of functional human primary follicular cell cultures were analyzed on Western blots using an antibodies to the amino-terminus (N-term) and to the carboxyl terminus (C-term) of Tie-2 as probes (Fig. 4). The cells were deprived of TSH for 72 h and incubated with varying concentrations of TSH for an additional 72 h. Antiserum to cytokeratin-8 was used to probe the same blot as a control of protein loading. TSH regulated the expression of Tie-2 receptor; maximal expression was seen at 100–300 mU/L TSH using either antibody. At TSH concentrations greater than this, the expression of Tie-2 returned to control levels. As well as full-length Tie-2 receptor of approximately 150 kDa, a fragment of 63 kDa was frequently found with the C-term antibody. No other bands were seen on the blots. Preadsorption of antibody with excess C-term antigen completely blocked immunolabeling of both bands (data not shown), confirming the specificity of the antibody. These experiments confirm the specificity of the antibodies for Tie-2 and show that the 63 kDa fragment is derived from the C-terminus, intracellular region of Tie-2.

TSH acts through cAMP to increase Tie-2 expression

Most of the effects of TSH are transduced through elevations in cAMP. To determine the role of this pathway in the regulation of Tie-2 expression, we used forskolin to activate adenylate cyclase and dibutyryl cAMP to mimic the effects of cAMP in human follicular cells. At a concentration of 10^{-5} mol/L forskolin there was an increase in the expression of Tie-2, and this was increased further at a concentration of 10^{-4} mol/L. Expression of Tie-2 was also increased using 5 mmol/L dibutyryl cAMP (Fig. 5).

FRTL-5 cells also express Tie-2

Figure 6 shows the regulation of Tie-2 expression by TSH, forskolin, and 8-bromo-cAMP in FRTL-5 cells on Western blots using the C-term antibody to Tie-2. Cells were cultured for 72 h in these conditions after a 72-h incubation in the absence of TSH. As in human cells, all of the cAMP agonists, including TSH, increased Tie-2 expression in FRTL-5 cells compared with control cells. The histogram *below* the figure shows the combined data from three separate experiments using the full-length receptor and shows a 7-fold increase with TSH (300 mU/L).

Tie-2 was detected in FRTL-5 cells as a full-length molecule of approximately 150 kDa as well as the fragment of 63 kDa. A higher molecular mass band of approximately 280 kDa was consistently seen with the C-term antibody. Although preincubation with excess C-term antigen blocked the labeling of this band, thus confirming its identity as Tie-2, the N-term antibody did not bind to this protein. We postulate that it is a covalently linked dimer of Tie-2 folded in such a way that the N-term antigenic site is concealed. The 135-kDa band is recognized by both antibodies, and excess C-term antigen blocks binding of the C-term antibody, but not the N-term antibody (data not shown).

Tie-2 and Ang-1 mRNA are also present in follicular cell culture

The expression of the mRNAs for Ang-1, Ang-2, and Tie-2 was analyzed by RNase protection assay as described in *Materials and Methods* and shown in Fig. 7. Tie-2 and Ang-1 mRNA was present in human follicular cells in culture. There was no Ang-2 mRNA detected in our preparations. Cells were deprived of TSH for 72 h and exposed to varying TSH concentrations for 24 h. There was increased expression of Tie-2 mRNA with TSH, reaching a maximum at 100–300 mU/L TSH. At higher TSH concentrations mRNA expression was inhibited. Densitometry was performed and corrected to a 28S riboprobe. Compared with the control there was a 36% increase in Tie-2, which fell to control levels after administration of 1 U/L TSH. Ang-1 increased by 48% at 100 mU/L, but did not fall to the control value at higher concentrations of TSH.

Discussion

We show for the first time that the Tie-2 receptor is expressed on human thyroid follicular epithelial cells as well as endothelial cells. The findings of Tie-2 expression on a clonal rat thyroid cell line FRTL-5 as well as expression of Tie-2

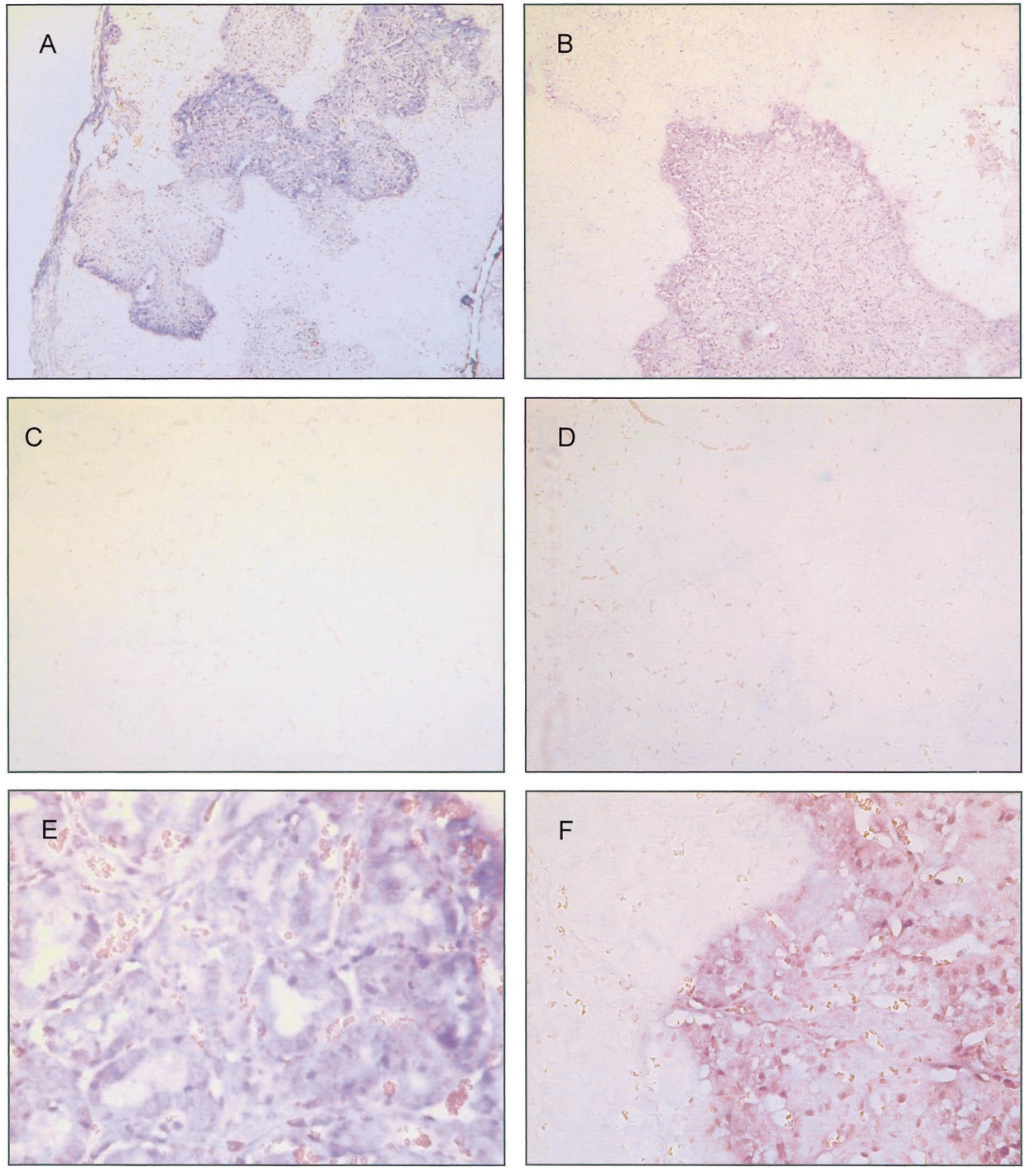

FIG. 3. *In situ* hybridization of rat thyroid after goiter was induced in rats with a low iodine diet and methimazole for 1 week. Thyroids were removed, and 5-μm sections were probed with digoxigenin-labeled probes. Tie-2 antisense (A) and Ang-1 antisense (B) at ×100 magnification. C, Tie-2 sense at ×100 magnification; D, Ang-1 sense at ×100. E, Tie-2 antisense at ×400; F, Ang-1 antisense at ×400 magnification.

mRNA in rat follicular cells during goiter induction support the findings in the human cells. Demonstrating Tie-2 expression in the FRTL-5 cell line obviates the concern that the Tie-2 found in primary culture is due to endothelial contamination. Tie-2 expression in adult tissues to date has been found only in endothelial cells (8, 9, 28), hemopoietic stem cells, and leukemic blast cells (11, 29). The endothelial restriction of Tie-2 is thought to be important for the biological actions of the angiopoietins. In embryogenesis, however, Tie-2 expression is found on several epithelial tissues, including lens epithelium and heart epicardium in rat embryos (28), as well as trophoblasts in the human placenta (19, 30). Conceivably, during goitrogenesis, the growth, differentiation, and reorganization required of the follicular cells may compare with those in embryogenesis.

We have demonstrated regulation of Tie-2 protein by TSH

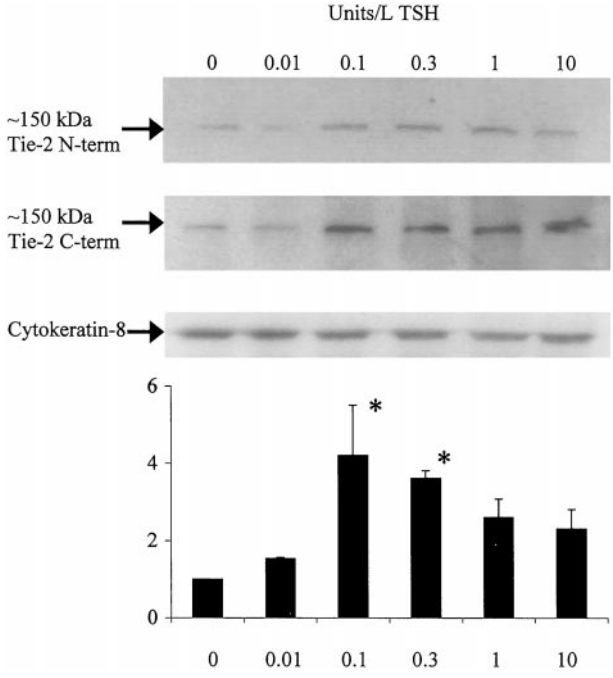

FIG. 4. Human primary follicular cells were incubated with varying concentrations of TSH for 72 h after a 72-h period of TSH deprivation. The total cell protein was analyzed by SDS-PAGE and probed for Tie-2 using antibodies raised to the C- or N-terminus. Immunoreactivity was found by both antibodies at 150 kDa, which corresponds to intact Tie-2. Total protein (100 μg) was loaded in each lane, and equal loading was confirmed by probing for cytokeratin-8. Densitometry is shown *below* for four experiments, with expression relative to the control shown, and significance was determined using Student-Newman-Keuls ANOVA. *, $P < 0.05$ *vs.* control.

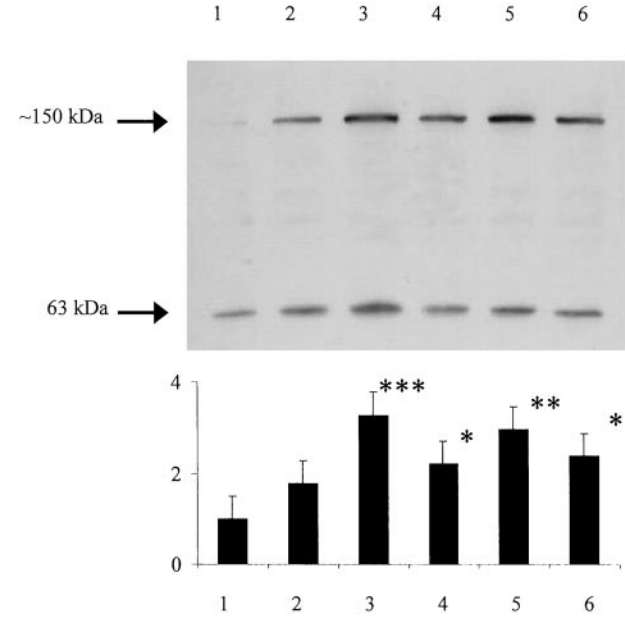

FIG. 5. Human primary follicular cells were incubated in control medium (lane 1), 100 mU TSH/L (lane 2), 300 mU/L TSH (lane 3), 10^{-5} mol/L (lane 4), 10^{-4} mol/L forskolin (lane 5),and 5 mmol/L dibutyryl cAMP (lane 6) for 3 days. Total cell protein was analyzed by SDS-PAGE and probed for Tie-2 (C-terminus). Both the full-length receptor and the 63-kDa fragment are shown. Total protein (100 μg) was loaded in each lane. Densitometry of three experiments is shown, with a representative blot. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (*vs.* control).

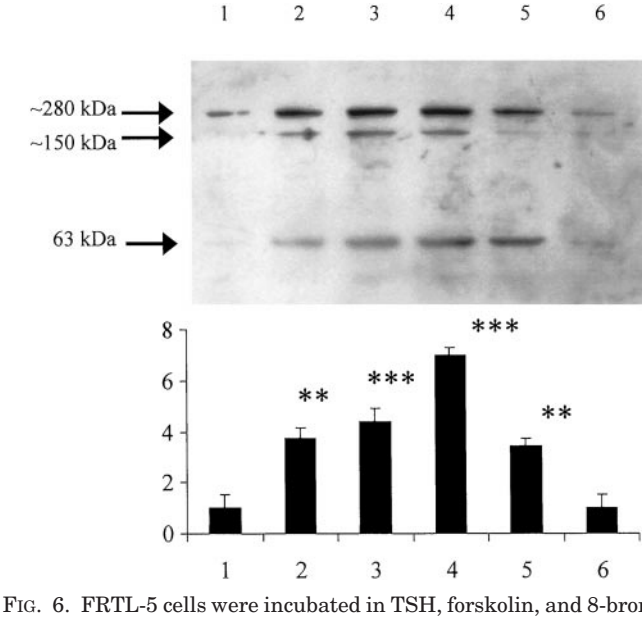

FIG. 6. FRTL-5 cells were incubated in TSH, forskolin, and 8-bromo-cAMP for 3 days. Lane 1, Control; lane 2, 0.01 mmol/L forskolin; lane 3, 0.1 mmol/L forskolin; lane 4, 300 mU/L TSH; lane 5, 1 mmol/L 8-bromo-cAMP; lane 6, control. Total cell protein was analyzed by SDS-PAGE and probed for Tie-2. Total protein (100 μg) was loaded in each lane. Densitometry of three experiments is shown, with a representative blot. **, $P < 0.01$; ***, $P < 0.001$ (*vs.* control).

and cAMP. Control of Tie-2 expression and the signaling pathways used are not yet well characterized. *In vivo* studies are limited and include reports of up-regulation of Tie-2 in breast cancer vasculature (22), wound healing in rats (31), and reperfusion after cerebral ischemia (32). No clear mechanism has been identified for these increases. *In vitro* there is little regulation of Tie-2 by hypoxia or cytokines, including VEGF, tumor necrosis factor-α, interleukin-1β, or FGF (33–35). The promoter for Tie-2 has been sequenced and partially characterized and contains several transcription factor binding sequences, including *ets*, SP-1, activating protein-1, and GATA-1 (36). Two isoforms of the *ets* family, NERF and NERF2, are expressed in endothelial cells, and appear able to up-regulate the Tie-2 promoter (37). Significant changes in the expression of Ang-1 and Ang-2 mRNA have been reported in response to cytokines and hypoxia. In bovine microvascular endothelial cells, Ang-1 is down-regulated in fibroblasts by cytokines, including platelet-derived growth factor and epidermal growth factor, and by hypoxia in a glioblastoma cell line (35). There are corresponding increases in Ang-2 levels in response to many of these stimuli, including VEGF and hypoxia (38) and FGF (33). No studies to date have examined regulation by cAMP of Tie-2 or the angiopoietins.

In our study the increase in Tie-2 mRNA was modest compared with the increase in protein when cells were stimulated with TSH. This may reflect the different time points used for isolation of mRNA (24 h after stimulation), whereas protein was measured after 72 h, or it may be that TSH stabilizes Tie-2 mRNA to increase protein formation. Alter-

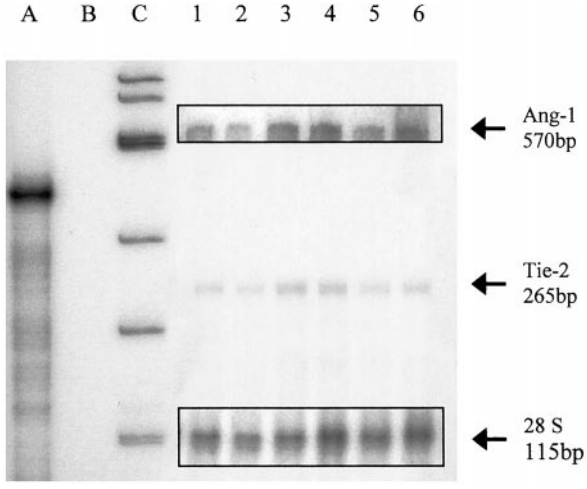

FIG. 7. RNase protection assay of Tie-2 and Ang-1 mRNAs in human thyroid primary cell cultures incubated with varying concentrations of TSH. Lane A, Undigested Tie-2 probe; lane B, yeast control; lane C, size markers. Lane 1, Control follicular mRNA; lane 2, 10 mU/L TSH; lane 3, 100 mU/L TSH; lane 4, 300 mU/L TSH; lane 5, 1 U/L TSH; lane 6, 10 U/L TSH. A 28S mRNA probe is shown as a marker of the RNA loaded.

natively reduced proteolysis, rather than increased synthesis, may mediate part of the regulation of the Tie-2 receptor by TSH, and further work is needed to confirm or refute this.

Although we do not know whether the stimulatory effects of TSH are direct or mediated through its ability to regulate the synthesis of another factor, our data showing positive regulation of Tie-2 expression by cAMP lead us to speculate that in endothelial cells, agents such as prostaglandins, which also act by elevating cAMP, may similarly regulate Tie-2 expression. In wound healing and inflammation where PG release is increased, this may lead to an increase in Tie-2 and a corresponding increase in neovascularization, and this is supported by the finding of increased total Tie-2 expression in healing wounds (31).

The maximum expression of Tie-2 in our cultures was found at 100–300 mU/L TSH, with inhibition at higher concentrations of TSH. This parallels the effects of TSH on uptake of ^{125}I (23), suggesting that the role of the Tie-2 receptor in thyroid follicular cells may be linked to their function. Tie-2 expression does not appear to be linked to follicle formation, because this varies directly with TSH concentration (data not shown), with no inhibition at higher TSH concentrations. Tie-2 could also function to sequester Ang-1 from the microenvironment in the thyroid, thereby modulating the angiogenic stimuli available to the endothelial cells. Tie-2 function may be regulated by proteolysis, because we found that Tie-2 was frequently proteolysed to a 63-kDa carboxylterminal fragment.

We show that Tie-2 protein expression was up-regulated in multinodular goiter in humans and, using *in situ* hybridization, that the mRNA was induced in goiters produced in rats. The patchy nature of the staining within the thyroid demonstrated both by immunohistochemistry on human surgical specimens and in the *in situ* studies of rat induced goiters is curious and may reflect the different sensitivity to TSH stimulation of different populations of follicular cells. Alternatively, it may reflect cell-cell communication through juxtacrine mediators.

We found that Ang-1 mRNA was present *in vitro* and in *vivo* and was increased in goiter formation in rats. At present we do not know whether Ang-1 produced by the follicular cells has an autocrine effect as well as a paracrine effect on endothelial cells. Whether Ang-1 acting through Tie-2 increases follicular cell growth, prolongs cell survival through its ability to activate akt (39), coordinates follicular and endothelial cell development, and/or regulates follicular cell function remains to be determined.

We did not find Ang-2 expression in the *in situ* studies of *in vivo* rat goiter or in the RNase protection assay of *in vitro* human thyrocytes. A recent study showed Ang-2 mRNA in tissue from thyroid cancers, but whether this was derived from endothelial or follicular cells was not determined (40). Ang-2, which opposes the actions of Ang-1, may not have a role in the remodeling of the vasculature that occurs in the developing thyroid, but whether it is expressed in goiter involution remains an interesting possibility.

The increase in Tie-2 and Ang-1 expression in human and rat goiter shows for the first time the involvement of the angiopoietin system in goitrogenesis. Modulation of angiogenesis through this pathway may be possible as a therapeutic treatment for goiter and other thyroid diseases.

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