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Am J Physiol Endocrinol Metab 284:E168-E176, 2003. First published 3 September 2002; doi:10.1152/ajpendo.00096.2002

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Isolation and characterization of human thyroid endothelial cells

VIMAL A. PATEL, ANN LOGAN, JOHN C. WATKINSON, SAAD UZ-ZAMAN, MICHAEL C. SHEPPARD, JAMES D. RAMSDEN, AND MARGARET C. EGGO
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Submitted 4 March 2002; accepted in final form 27 August 2002

Patel, Vimal A., Ann Logan, John C. Watkinson, Saad Uz-Zaman, Michael C. Sheppard, James D. Ramsden, and Margaret C. Eggo. Isolation and characterization of human thyroid endothelial cells. *Am J Physiol Endocrinol Metab* 284: E168–E176, 2003. First published September 3, 2002; 10.1152/ajpendo.00096.2002.—From collagenase digests of human thyroid, endothelial cells were separated from follicular cells by their greater adherence to gelatin-coated plates. Endothelial cells were further purified using fluorescence-activated cell sorting, selecting for cells expressing factor VIII-related antigen. Isolated cells were negative for thyroglobulin and calcitonin when examined by immunostaining. The receptor for the angiopoietins, Tie-2, was expressed by the cells, and expression was increased by agents that elevate cAMP. Nitric oxide synthase (NOS) 3, the endothelial form of NOS, was expressed by the cells and similarly regulated. Cells responded strongly to the mitogen fibroblast growth factor (FGF)-2 in growth assays but only weakly to vascular endothelial growth factor (VEGF). VEGF was, however, able to stimulate nitric oxide release from the cells consistent with their endothelial origin. The FGF receptor (FGFR1) was full length (120 kDa) and immunolocalized to the cytosol and nucleus. Thyrotropin (TSH) did not regulate FGFR1, but its expression was increased by VEGF. Thrombospondin, a product of follicular cells, was a growth inhibitor, but neither TSH nor 3,5,3′-triiodothyronine had direct mitogenic effects. Thyroid follicular cell conditioned medium contained plasminogen activator activity and stimulated the growth of the endothelial cells, but when treated with plasminogen to produce the endothelial-specific inhibitor, angiostatin, growth was inhibited. Human thyroid endothelial cell cultures will be invaluable in determining the cross talk between endothelial and follicular cells during goitrogenesis.

Tie-2; thyrotropin; vascular endothelial growth factor; fibroblast growth factor receptor-1; angiostatin; thrombospondin; plasminogen activators

ANGIOGENESIS IS THE proliferation of endothelial cells and their organization into new blood vessels. The endothelium plays a vital step in the development of a number of proliferative pathologies (21), and the ability to regulate its activity offers new treatment modalities for aberrant growth. During goitrogenesis induced in rats, angiogenesis was shown to be rapid and to precede follicular cell growth (47). An endothelial chemoattractant was isolated but not identified (17), and later, fibroblast growth factor (FGF)-2, a known angiogenic growth factor, was isolated from thyroids (11). We have shown that elevations in follicular cell production of FGF-2 and reductions in thrombospondin (TSP-1) accompany the angiogenesis occurring during goitrogenesis in the rat (31) and that elevations in FGF-1, FGF-2, and FGF receptor (FGFR)-1 expression also occur in human goiter (9, 42). The mitogenic and angiogenic activity of FGF-2 suggests a role in the pathogenesis of goiter, malignant or otherwise (3).

Vascular endothelial growth factor (VEGF) may also play a role in thyroid angiogenesis. Three VEGF receptor subtypes have been described, restricted in expression to the endothelium, which may in part explain the multiplicity of biological actions ascribed to VEGF in the literature (44, 32). VEGF was initially discovered as a tumor-derived factor, which increased microvascular permeability. Subsequently, the protein was found to exhibit mitogenic effects on endothelial but not other cell types (12, 18, 28). In normal thyroids, VEGF was found to be present within the follicular cells (38) and shown to be secreted in response to thyrotropin (TSH) from a thyroid cancer cell line in vitro (40). VEGF was also elevated within goiters and, in the FRTL-5 rat thyroid cell line, VEGF was found to significantly reduce the ability of TSH to increase ^{125}I uptake (46).

The newest members of the angiogenic growth factor family are the angiopoietins (Ang-1 and Ang-2) and their receptor, Tie-2. The receptor, which is a receptor tyrosine kinase, is almost exclusively expressed on endothelial cells. The angiopoietins cooperate with VEGF and are thought to be important in later stages of vessel growth, such as angiogenic outgrowth, vessel remodeling, and maturation, and may be involved in communication of endothelial cells with the mesenchyme (22).

The anti-angiogenic factor, TSP-1, is secreted by thyroid epithelial cells from their basolateral pole, and its production is negatively regulated by TSH (34). TSP-1 is postulated to be involved in the organization of thyroid follicles but may act on the vasculature

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surrounding them. The production of TSP-1 by endothelial cells may influence the cell-to-cell or the cell-to-matrix interactions needed for formation of capillary tubes (35). Another anti-angiogenic factor is angiostatin, a fragment of plasminogen formed by the action of proteases, such as plasminogen activators (30). Thyroid cells are known to be active in secreting plasminogen activators and other proteases (27).

Endothelial cells from different sites show considerable heterogeneity in their immunological and metabolic properties (15, 43). A recent paper showed that some endocrine tissues produced an angiogenic mitogen that is selective for endocrine gland endothelium, confirming that endothelium from different sites differs (25). The thyroid endothelium was not, however, investigated in this study. To investigate angiogenesis in the thyroid, isolation and characterization of human thyroid endothelial cells are therefore necessary. The purpose of this study was to isolate and characterize normal thyroid endothelial cells and to investigate the relationships between thyroid follicular cells, which produce FGF-2, VEGF, proteases, thyroid hormones, etc., which may play a role in the angiogenic response. We used the property of endothelial cells to attach to gelatin as a crude separation before using fluorescence-activated cell sorting (FACS), with antibodies specific for endothelial cells, to retrieve a pure population.

EXPERIMENTAL PROCEDURES

Unless otherwise stated, reagents were obtained from Sigma Chemical (Poole, UK).

Antisera. The antisera used for FACS and immunohistochemical and Western analyses were as follows. Rabbit polyclonal anti-factor VIII-related antigen (Dako, Copenhagen, Denmark) is a specific endothelial cell surface marker. Rabbit polyclonal anti-human thyroglobulin antiserum was a kind gift of Elizabeth Black (Dept. of Medicine, University of Birmingham). Rabbit polyclonal anti-calcitonin antiserum was from ICN Biologicals (Costa Mesa, CA), and rabbit polyclonal anti-human FGF-2 sera was a kind gift of Dr. A. Baird. A rabbit polyclonal anti-human FGFR1 sera raised against the NH_2-terminal extracellular region of the receptor was from Promega (Southampton, UK). Tie-2 antibody was made in rabbits to the COOH-terminus of Tie-2 (Santa Cruz, CA).

Human thyroid endothelial cell isolation. Human thyroid glands were obtained at surgery from multinodular goiters. The tissue was incubated in 0.1% type II collagenase (Worthington) overnight at room temperature. The digested tissue was passed through a 200-μm filter to remove undigested material, and the collagenase was removed by centrifugation at 360 *g* at 4°C for 10 min. The supernatant was discarded, and the cellular pellet was resuspended in DMEM supplemented with 10% newborn bovine calf serum (NCS) containing 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were plated on dishes coated with 1% gelatin. Cells were incubated for up to 1 h and examined microscopically every 10 min. When thyroid follicles began to attach to the dishes, the cell suspension was removed, and the dishes were washed extensively and vigorously with Hanks' balanced salt solution (HBSS) to remove adherent follicles. Single cells remaining adherent after these washings were incubated with fresh endothelial cell medium that consisted of a 1:1 mixture of 10% NCS in DMEM and conditioned media from JEG cells supplemented with 1 ng/ml FGF-2, 17.5 U/ml heparin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 250 μg/ml nystatin. This medium is a modification of that described by Folkman et al. (13). JEG cells are a human cell line derived from a choriocarcinoma and are cultured in DMEM containing 10% FBS. Cultures were incubated in humidified incubators with 5% CO_2-95% air, and the media were replaced every 2 days. The human thyroid endothelial cells used for the experiments reported in this article were obtained from an individual with multinodular goiter who was euthyroid.

FACS analysis. When the cells had grown to confluence, they were removed from the dishes with 0.1% trypsin in calcium- and magnesium-free HBSS and washed with HBSS by suspension and centrifugation. The cells were suspended with rabbit anti-factor VIII-related antigen (DAKO; a specific, cell-surface marker for endothelial cells) in DMEM with 1% NCS for 1 h in the incubator. The cells were washed and incubated with FITC-conjugated anti-rabbit secondary antibody for 1 h diluted in DMEM-1% NCS. After being washed, the cells were sorted on the FACS machine. The percentage of immunopositive cells was determined, and the isolated cells were grown on gelatin-coated plates with media. Cells grew slowly in clumps, but colonies were visible to the naked eye after 8 wk of culture. At this point, cultures were passaged with 0.1% trypsin in Tris-EDTA (in mM: 10 Tris, 130 NaCl, and 5 EDTA, pH 7.2) on gelatin-coated plates in endothelial cell medium. The cells passaged well with little cell loss and could be maintained in continuous culture. Early passaged cells were frozen and returned to culture with good viability.

Assessment of cell growth. Cells were passaged with 0.1% trypsin in Tris-EDTA on 24-well, 2-cm^2 gelatin-coated plates and left to adhere and recover for 48 h in DMEM-10% NCS. Cells were washed and grown in serum-free DMEM for 24 h, after which the cells were incubated in serum-free media containing the factor to be assayed for a further 48 h. The cells were washed in Ca^{2+}- and/or Mg^{2+}-free HBSS, and 1 ml HEPES buffer and two drops of Zaponin (Coulter Electronics, Luton, UK) were added. The cells were left at 37°C until the cytoplasmic membrane lysed, leaving the nuclei intact. The suspension of nuclei was then transferred to an Accuvette (Appleton Woods, Birmingham, UK) containing 9 ml formol saline (0.5% formalin, 0.9% NaCl) to fix the nuclei. A Coulter Counter (Coulter Electronics) was used to count the number of nuclei in the sample two times. All experiments were performed on quadruplicate wells, and three replicate assays were used for statistical analysis.

Immunofluorescent labeling of cells in monolayer. Cells were grown on a multiwell noncoated slide (The Binding Site, Birmingham, UK). The cells were air-dried (at which point they can be stored at 4°C) and fixed with 100% acetone at 4°C for 2 min. The slides were washed in PBS (10 mM phosphate, 150 mM NaCl, pH 7.4) for 15 min and incubated at room temperature for 30 min with a nonimmune blocking serum (from the species in which the secondary antibody is made) diluted in PBS to block the nonspecific binding sites. The cells were washed and incubated with primary antisera diluted with PBS for 1 h at room temperature. The cells were washed again and incubated with PBS-diluted fluorescein-conjugated secondary antibody for 30 min at room temperature. The cells were washed and counterstained with propidium iodide (1 μg/ml) for 2 min to identify nuclei, after which the cells were washed and mounted using 2.5% 1,4-diazobicyclo-(2.2.2)-octane in 80% glycerol to prevent bleaching of fluorescence. Immunofluorescence was viewed using a microscope equipped for epifluorescence, with filters for fluorescein and/or rhodamine.

Nitric oxide synthesis. The production of nitric oxide (NO) synthesis after VEGF stimulation was measured using a Sievers 280 NO Analyzer. The human endothelial cells were cultured in serum-free medium containing FGF, heparin, and antibiotics as described earlier for 24 h before the start of the experiment. VEGF of varying concentrations was added directly to quadruplicate wells, and incubation continued for 1 h as described previously (5). Media were removed and frozen at −70°C until assay. Duplicate background control wells without cells were incubated as described to account for NO release in the media itself.

Western blotting. The human thyroid endothelial cells were grown in serum-free media for 24 h and cultured in agonist for a further 48–72 h. The cells were lysed in 1% SDS and passed through a syringe needle to shear viscous DNA. The protein concentration of the sample was determined by protein assay (DC kit; Bio-Rad Laboratories, Hemel Hempstead, UK) to ensure equal loading of gels. An equal volume of buffer (125 mM Tris, pH 6.8, 20% glycerol, 4% wt/vol SDS, 10% β-mercaptoethanol, and 0.001% wt/vol bromphenol blue) was added, and samples were heated at 95°C for 2 min. The protein samples were separated on a 4% polyacrylamide stacking gel containing 0.125 M Tris, pH 6.8, 0.1% (wt/vol) SDS, and a 12% polyacrylamide running gel containing 0.4 M Tris, pH 8.8, and 0.1% (wt/vol) SDS. The running buffer contained 25 mM Tris, 0.2 M glycine, and 1% SDS. In addition to the samples, protein standards of known molecular weight were also run. The proteins on the SDS gels were electrophoretically transferred to Immobilon-polyvinylidene difluoride transfer membranes (Millipore, Bedford, UK). Proteins were detected by use of selective antibodies (see below) and visualized by enhanced chemiluminescence (Amersham). Autoradiographic signal intensity was quantified by densitometry in the linear range of film exposure where appropriate.

Thyroid cell conditioned medium: preparation and the effects on thyroid endothelial cells. Thyroid follicular cell conditioned medium was collected from thyroid cells isolated from a multinodular goiter. Cells had been in culture for 10 days and were cultured in serum-free medium containing antibiotics, insulin (100 ng/ml), and TSH (0.3 mU/ml), as described previously (10). The medium was conditioned by the thyroid cells for 72 h. Production of angiostatin using this medium was essentially as described by Gately et al. (16). Plasminogen (100 μg/ml) was added to one-half of the conditioned medium, and both aliquots were incubated with L-cysteine (100 μM) for 24 h at 37°C. For the growth assay, 250 μl of endothelial cell medium, made with heat-treated serum to denature active proteases and plasminogen, and 250 μl thyroid conditioned medium treated with or without plasminogen or an equivalent volume of the control medium were used. Control medium comprised of Coon's modification of Ham's F-12 medium supplemented with 0.1 μg/ml insulin, 0.3 mU/ml TSH, 100 U/ml penicillin, and 100 μg/ml streptomycin. This was incubated for 24 h at 37°C in the presence and absence of plasminogen and L-cysteine, corresponding to the different conditions of the thyroid medium. Medium was supplemented with radioactive [*methyl*-^{3}H]thymidine (sp act 80 Ci/mmol; Amersham) at a final concentration of 0.5 μCi/well. The endothelial cells were incubated for 24 h in the different conditions at 37°C. After incubation, the medium was removed, and the endothelial cell layer was washed with HBSS. One milliliter of 6% TCA was added to each well at 4°C for 30 min. The TCA was removed, and 1 ml of fresh 6% TCA was added to each well for a final wash. The cell layer was solubilized in 1.0 M NaOH, and radioactivity was determined in a liquid scintillation counter.

Plasminogen activator assays. Conditioned medium from thyroid follicular cells from multinodular goiters, incubated for 72 h in varying concentrations of TSH as described previously (10), was collected, and plasminogen activator assays were performed as described previously (27) using human urokinase (Calbiochem, CN BioSciences, Beeston, Notts, UK) of activity 80,000 IU/mg as standard.

Statistics. Statistical analysis was performed using Student's *t*-test, and $P < 0.05$ was selected as the level of significance.

RESULTS

Endothelial cell isolation. Using FACS analysis, we found that, routinely, 20% of the cells that were grown on the gelatin-coated tissue culture wells were endothelial cells, 4% were thyrocytes, and 5% were C cells ($n = 5$). The remaining cells were not characterized but were probably derived from the mesenchyme. The factor VIII-related antigen-positive cells collected by FACS were grown on gelatin-coated substratum. By phase-contrast microscopy, the human thyroid endothelial cells were observed to have an epithelial morphology consistent with microvascular endothelial cells rather than a "cobblestone" appearance that is indicative of large-vessel endothelial cells such as aortic or umbilical vein endothelial cells (1, 19). The doubling time of these cells was 24–36 h.

Early passage cells failed to divide under serum-free conditions and required gelatin for growth. However, later-passage cells were able to survive on normal tissue culture plastic and were able to withstand serum-free culture for up to 4 days. All experiments described were performed on these later-passage cells. Figure 1 shows a micrograph of the human thyroid endothelial cell isolate.

Immunostaining to confirm endothelial cell origin. To confirm the endothelial cell characteristics of the isolated cell strain, we performed immunostaining for factor VIII-related antigen, thyroglobulin, and calcitonin, as shown in Fig. 2*A*. Cells were positive for factor VIII-related antigen but negative for thyroglobulin and

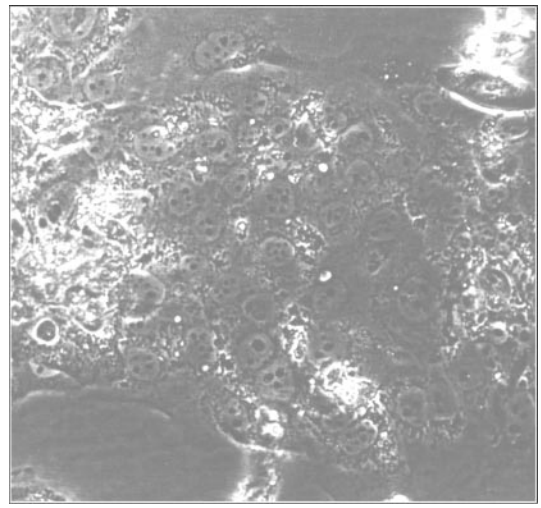

Fig. 1. Phase-contrast micrographs of a human thyroid endothelial cell isolate growing on gelatin; ×100 magnification.

calcitonin, indicating successful isolation of a pure population of human thyroid endothelial cells. Thyroid follicular cells were also stained for these antigens and were only positive for cytoplasmic thyroglobulin. Figure 2, *B* and *C*, shows images taken from the confocal microscope of immunofluorescence staining for FGF-2 and FGFR1, respectively, in the isolated human endothelial cells. Cells expressed FGF-2 within the cytoplasm and nucleus, and perinuclear staining was clearly evident in some cells. Cells reacted strongly with antisera to the NH_2-terminus (extracellular domain) of FGFR1. The FGFR1 immunoreactivity was present in the cytoplasm but also within the nuclear region of these cells, as indicated by the yellow staining that represents the merging of the green fluorescence (antigen) with the red propidium iodide (DNA).

Growth of thyroid endothelial cells to angiogenesis regulators TSH and 3,5,3′-triiodothyronine. The responsiveness of isolated human thyroid endothelial cells to the angiogenesis regulators TSH and 3,5,3′-triiodothyronine (T_3) were assessed in growth assays. Figure 3*A* shows the results with FGF-2, VEGF, and TSP-1. FGF-2 was a mitogen to the endothelial cells at concentrations of 1, 10, and 100 ng/ml. VEGF was significantly mitogenic at the low concentration of 0.01 ng/ml and also at 1 ng/ml. TSP-1 was growth inhibitory at statistically significant levels of 0.1, 1, and 10 ng/ml. Figure 3*B* shows the effects of increasing concentrations of TSH or T_3 on endothelial cell growth. Neither of the agents, at any concentration, showed an effect on endothelial cell growth.

Plasminogen activator activity in human thyroid cell conditioned medium: effect of TSH. Plasminogen activator activity was readily detectable in human thyroid cell-conditioned medium. Cells incubated in medium without TSH had an activity of 7.7 ± 0.2 IU/ml. When TSH was included in the culture medium for 72 h, plasminogen activator activity fell to 1.2 ± 0.2 IU/ml.

Effect of thyroid follicular cell-conditioned medium incubated with or without plasminogen on thyroid endothelial cell growth. Thyroid endothelial cell growth was stimulated by thyroid follicular cell-conditioned medium, as shown in Fig. 4. After overnight incubation with plasminogen and L-cysteine, the growth stimulatory effect of the conditioned medium was lost, and growth was inhibited compared with control, incubated identically but with medium not conditioned by thyroid follicular cells. In this control experiment, plasminogen itself stimulated growth of the thyroid endothelial cells, although this was not found in all assays performed on these cells. Similar results were found with human foreskin fibroblasts treated plasminogen and probably relate to the known mitogenic effects of proteases (26). Regardless of this, the growth stimulatory effects of the conditioned medium were consistently reduced down to control levels when incubated with plasminogen and L-cysteine. This inhibitory effect was not seen when this medium was incubated with FRTL-5 cells, which are an epithelial rat thyroid cell line (data not shown).

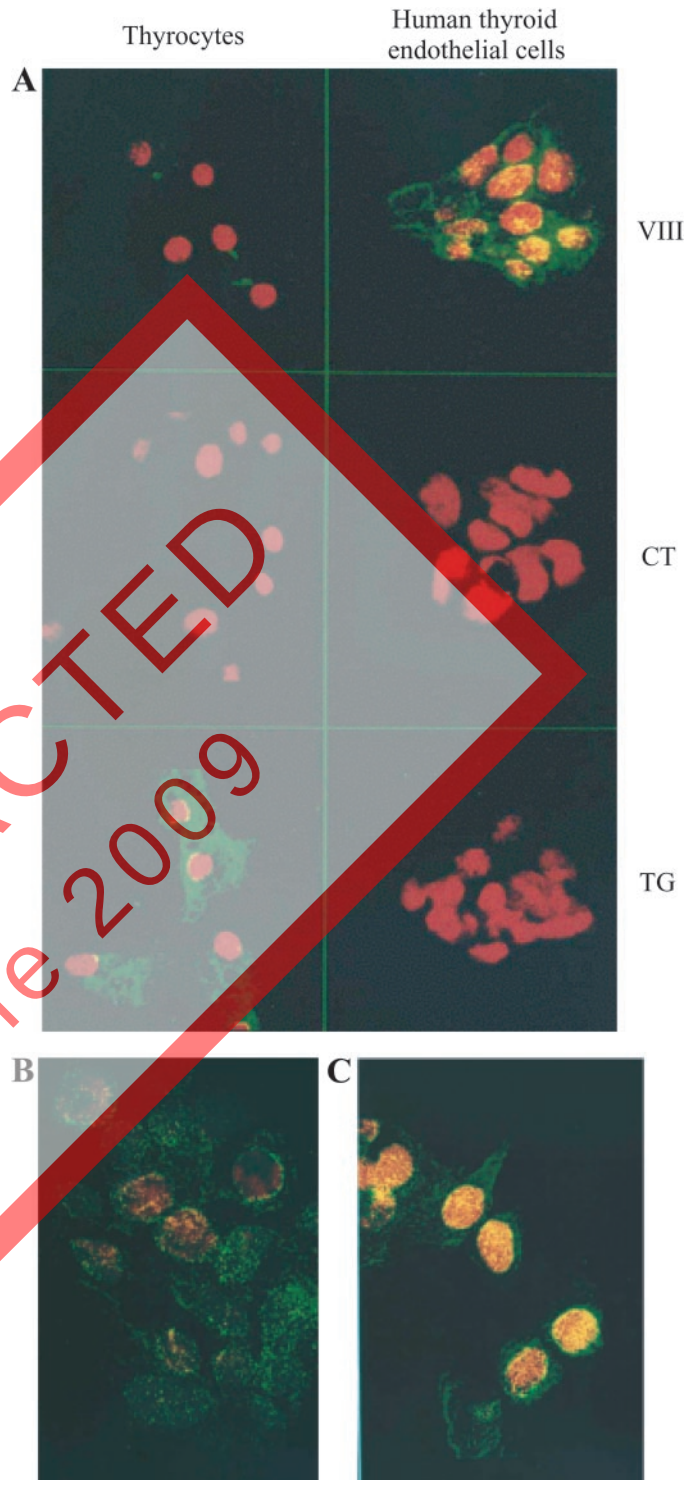

Fig. 2. Confocal images of immunofluorescent labeling. *A*: micrograph of thyrocytes and thyroid endothelial cells stained for thyroglobulin (TG; thyrocyte specific), calcitonin (CT; C cell specific), and factor VIII-related antigen (VIII; endothelial cell specific). Green, positive immunoreactivity; red, propidium iodide nuclear counterstaining; yellow, colocalization of both fluorescent probes. *B*: immunofluorescent labeling for fibroblast growth factor (FGF)-2. *C*: FGF receptor (FGFR)-1 (NH_2-terminal specific antisera) on thyroid endothelial cells. Nuclear immunolocalization is indicated by yellow staining.

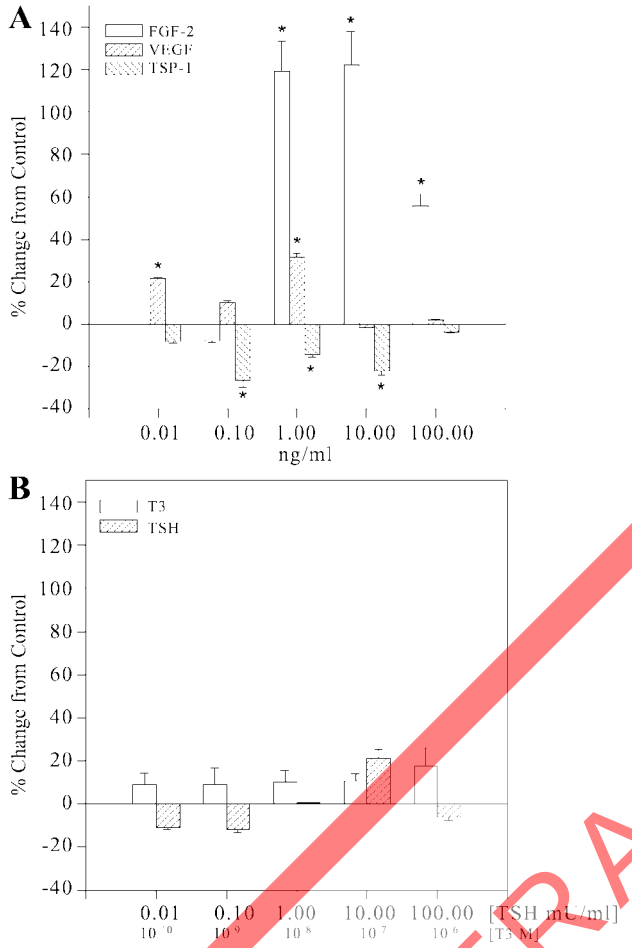

Fig. 3. *A*: growth assay for FGF-2, vascular endothelial growth factor (VEGF), and thrombospondin (TSP-1; administered in ng/ml) on human thyroid endothelial cells. *B*: growth assay for 3,5,3′-triiodothyronine (T_3; administered in molar concentration) and thyrotropin (TSH; administered in mU/ml) on human thyroid endothelial cells. Values expressed as percentage of control levels and are shown as means ± SD from 3 experiments. $^*P < 0.05$ vs. control.

NO production. When incubated with VEGF for 60 min, as described in EXPERIMENTAL PROCEDURES, increased NO was found in the medium of the endothelial cells (Fig. 5). All concentrations tested gave significant increases; however, the stimulation was greatest at lower concentrations of 1–3 ng/ml and decreased at higher concentrations ($n = 4$).

Western blotting of FGFR1, Tie-2, and NO synthase-3. Western blotting for FGFR1 showed that the full-length receptor of 120 kDa was expressed in all samples when blots were probed using antibody to the NH_2-terminus of FGFR1. There was no apparent regulation of the full-length FGFR1 protein expression by TSP-1 when compared with internal controls (data not shown). However, VEGF was found to increase FGFR1 expression, with maximum protein expression observed at 1 ng/ml VEGF (Fig. 6*A*), which was confirmed by densitometry (Fig. 6*B*). Equal amounts of protein were loaded on the gel, as assessed by protein assay. Coomassie blue staining of the samples on the gels also confirmed equivalent loading.

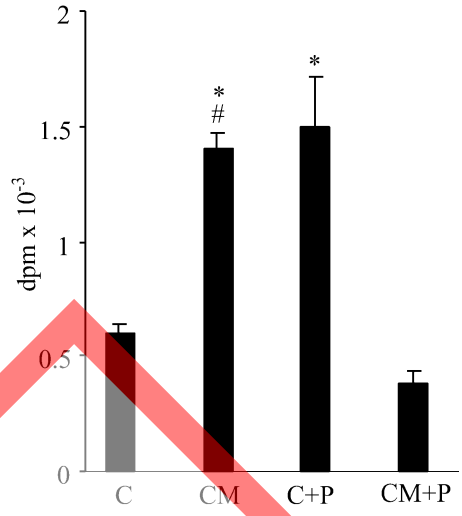

Fig. 4. Thyroid endothelial cell thymidine uptake in the presence or absence of normal thyroid cell-conditioned medium with or without plasminogen and L-cysteine. Unconditioned thyroid cell medium (C), conditioned medium (CM), C + plasminogen (P) and L-cysteine, CM + P and L-cysteine were added to 6 wells of endothelial cells. Cells were incubated with 0.5 μCi [*methyl*-^{3}H]thymidine for 24 h. Data are means + SE; $n = 3$ experiments. $^*P < 0.01$ vs. C. $\#P < 0.001$ vs. CM + P.

Western blotting was used to determine the regulation of the expression of the endothelial cell-specific protein Tie-2, as shown in Fig. 7. The effects of TSH on Tie-2 expression in endothelial cells were examined, and no significant regulation was found, consistent with the absence of direct effects of TSH. Expression of full-length Tie-2 of ~150 kDa was increased when cells were incubated with cell-permeable cAMP analogs (8-bromo-cAMP or dibutyryl-cAMP) or with forskolin, which activates adenylate cyclase. In the absence of these agents, Tie-2 expression was barely detectable.

The thyroid endothelial cells were further characterized by investigating the endothelial NO synthase (NOS)-3 levels. Figure 7*B* shows a representative

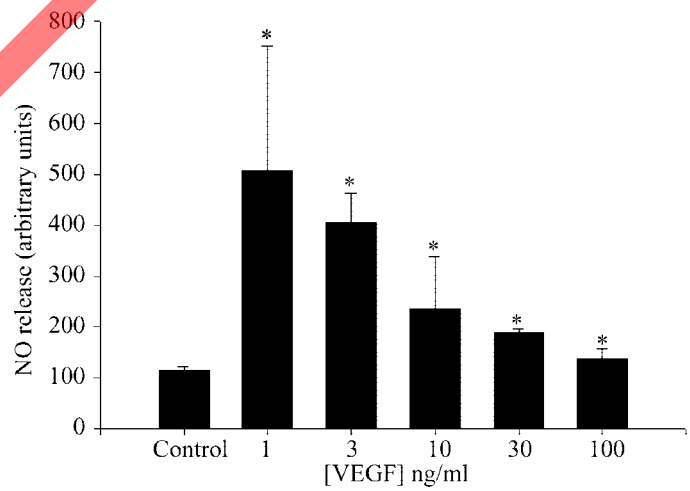

Fig. 5. Effects of increasing concentrations of VEGF on nitric oxide (NO) release from thyroid endothelial cells. Bars represent percent change from control levels and show means ± SD. All values are significant at $^*P < 0.05$.

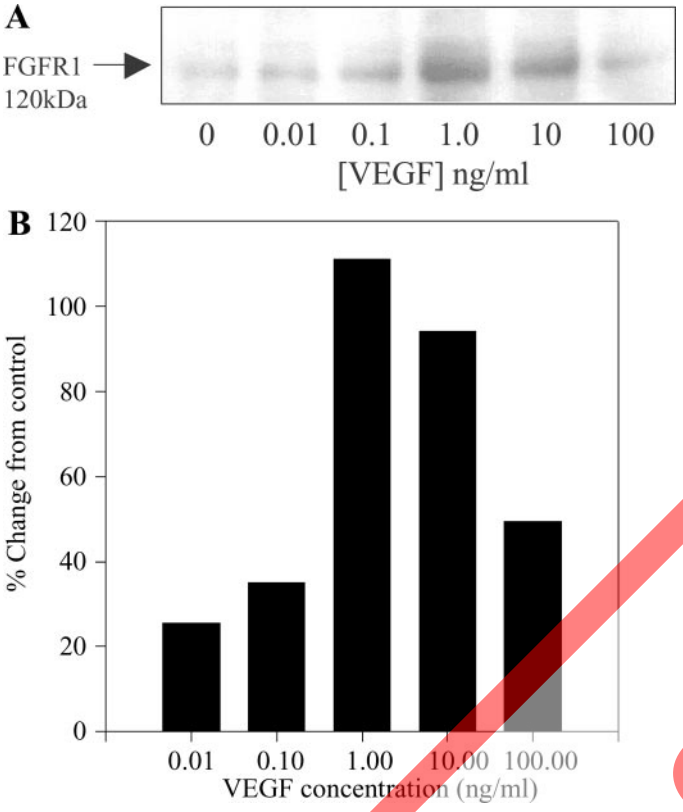

Fig. 6. Western blot analysis on human endothelial cell total protein lysates. *A*: VEGF dose responses probed for FGFR1. The blots show a full-length FGFR1 of 120 kDa detected with antisera specific to the NH_2-terminus. *B*: densitometry of the VEGF dose-response Western blot that shows increased expression of FGFR1 in response to VEGF. Equal amounts of protein were loaded in each lane.

Western blot of Tie-2 and NOS-3 expression in human thyroid endothelial cells treated with increasing concentrations of dibutyryl-cAMP. We observed a dose-dependent increase in the expression of both proteins. Tie-2 expression was observed at 30 μM and NOS-3 expression at 100 μM.

DISCUSSION

We have described a method for the isolation of a cell strain of microvascular thyroid endothelial cells using FACS, selecting for cells expressing factor VIII. This is an extension of the original protocol that uses the preferential affinity of endothelial cells for gelatin (13). Recently, methods have become available for the isolation of endothelial cells using beads coated with antibodies to Ulex europeus agglutinin-1 or CD-34 (14, 19, 20) primarily from vascular-rich tissues, a method that may prove useful for laboratories wishing to attempt this procedure from primary cultures. Antibodies to other endothelial cell-specific antigens could also be used (27). In our study, isolated cells were grown up and passaged and were found to be more robust than their parent cells because they no longer required gelatin for cell attachment to the culture dish. This cell strain has now been in continuous cell culture for 3 yr. Myometrial microvascular endothelial cells have been shown to have a stable phenotype over 14 passages when cultured in human serum (14), but the ECV304 cells, which were thought to be derived from human umbilical vein endothelial cells, have recently been shown to be a bladder cancer-derived epithelial cell line (4).

Cells are able to withstand serum-free conditions for several days, suggesting that they may be producing autocrine growth factors that promote cell survival. FGF-2 was expressed by the cells, consistent with data showing immunoreactivity in endothelial cells from other tissues (29). Because the cells also express FGFR1, this FGF-2 may act in an autocrine manner to modulate endothelial cell survival, movement, and function (23, 39). However, the expression of FGF-2 was low both by immunostaining and by Western blotting (data not shown), suggesting that paracrine FGF-2, primarily from the thyroid follicular cells (3, 7, 11, 31, 42), is the more likely modulator in vivo.

FGF-2 was a mitogenic factor to thyroid endothelial cells, but at 100 ng/ml FGF-2 levels there was a reduction in the maximal growth observed at lower concentrations. This bell-shaped response is a characteristic of FGF-2 and may be the result of receptor downregulation, receptor desensitization (33, 45), or negative cooperativity, as described for the insulin receptor (6). We also found cytoplasmic and nuclear localization of FGFR1, which has been observed in other cell types (24, 41), but its precise role in controlling growth of endothelial cells is unclear.

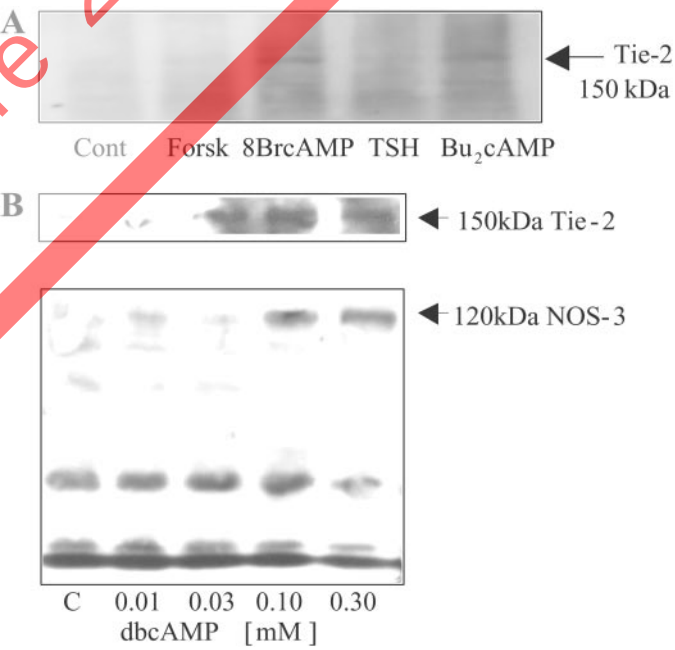

Fig. 7. *A*: Western blot analysis of Tie-2 expression in human thyroid endothelial cells in response to 10^{-5} M forskolin (Forsk), 10^{-3} M 8-bromo-cAMP (8BrcAMP), 3×10^{-10} M TSH, or 10^{-3} M dibutyryl-cAMP (Bu_2-cAMP). Equal amounts of protein were loaded in each lane. *B*: Western blot analysis of Tie-2 and nitric oxide syntheses (NOS)-3 expression in human thyroid endothelial cells in response to increasing concentrations of the cAMP analog dibutyryl-cAMP. Equal amounts of protein were loaded in each lane.

The effects of TSP-1 on endothelial cell growth have been less well characterized than those of FGF-2. We found that, in thyroid endothelial cells, exogenous TSP-1 was a growth inhibitor, but its effects were not potent. One other study has shown that TSP-1 inhibited human umbilical vein endothelial cell growth by 65% after 6 days and that of pulmonary artery endothelial cells by 72% after 72 h (2). Thyroid endothelial cells showed a 30% growth inhibition at 72 h. We have previously examined TSP-1 expression in goiters induced in rats (26) when angiogenesis is occurring in the hyperplastic gland and found that TSP-1 expression was upregulated within the follicular cells during the early angiogenic response. Later in the angiogenic response, TSP-1 expression was decreased, which may provide an extra stimulus for the angiogenic response initiated by the angiogenic growth factors FGF-2 and VEGF.

An ELISA was performed to determine the levels of VEGF and FGF-2 that are produced by thyroid follicular cells in culture; 10^6 cells were found to produce 6 ng of VEGF per day and 6 pg of FGF-2 per day, showing that the major angiogenic factor produced by thyroid follicular cells is VEGF.

We found that Tie-2, the receptor for the angiopoietins, was expressed on the thyroid endothelial cell strain. This receptor was thought to be exclusive to the endothelium, but we have shown that Tie-2 is also found on thyroid follicular cells (36). On follicular cells we found that Tie-2 expression is regulated by TSH and cAMP. In the thyroid endothelial cells, TSH did not regulate Tie-2, consistent with the limited distribution of this receptor, but activation of adenylate cyclase by forskolin or elevations in cAMP by cell-permeable cAMP analogs was effective. These data confirm the regulation of expression of Tie-2 by cAMP found in the follicular cells. One of the possible endogenous stimulators of cAMP production in human thyroid endothelial cells may be PGE_2. Elevations in cAMP induced by TSH in follicular cells could conceivably act as juxtacrine regulators of Tie-2 in the endothelial cells. We have also shown that thyroid follicular cells synthesize Ang-1 (36), thus providing the ligand for Tie-2 on the endothelial cells.

We could find no direct effects of TSH or T_3 on thyroid endothelial cell growth or on the expression of FGFR1. This suggests that the angiogenic events occurring during goitrogenesis are not directly under the control of the increased TSH levels or the result of the effects of TSH on T_3 and thyroxine production from thyroid follicular cells. However, TSH may indirectly regulate angiogenesis by regulating the secretion of angiogenic (VEGF, FGF-2, angiopoietins) or antiangiogenic factors (TSP-1, angiostatin). TSH may also regulate proteolytic activity. Proteases could release FGF-2 from storage in the extracellular matrix, activate latent forms of other growth factors, or produce antiangiogenic factors from circulating plasminogen. In this study, we show that thyroid cell-conditioned medium contained considerable amounts of plasminogen activator activity that was decreased by TSH treatment. Conditioned medium from thyroid cells stimulated the growth of endothelial cells, but, after incubation with plasminogen and L-cysteine, growth inhibitory effects were seen. This is likely to be because of the generation of angiostatin, known to be a specific inhibitor of endothelial cell growth (37). The inhibition of plasminogen activator activity by TSH would result in decreased production of angiostatin, which would permit endothelial cell growth essential for goitrogenesis. These studies demonstrate the cross talk between the cells comprising the thyroid and show how the balance between angiogenic and antiangiogenic factors can be regulated.

We found NO release from the thyroid endothelial cells after VEGF stimulation. In all of our assays of VEGF action, a very sensitive response was found (maximum 1 ng/ml), and higher VEGF concentrations were less effective. This is consistent with the endothelial origin of the cells. NO is generated by three known isoforms of NOS. The endothelial isoform, NOS-3, was shown to be abundantly expressed in the rat thyroid gland, and its expression was increased during goitrogenesis in rats (7, 8). This increase was within both the vasculature and the thyroid follicular cells and correlates with the increased VEGF that accompanies goitrogenesis (46). The localization of NOS-3 is consistent with NO having a role within vascular control of the thyroid and in thyrocyte function and/or growth (7). We found NOS-3 expression in the endothelial cells described in this study.

The increase of FGFR1 expression in thyroid endothelial cells observed after VEGF exposure suggests that VEGF may act in cooperation with FGF-2 on these endothelial cells. Because VEGF is produced in 100-fold excess compared with FGF-2 in thyroid follicular cells, but is a less potent mitogen to thyroid endothelial cells than FGF-2, this FGFR1 regulation by VEGF may be an important mechanism for thyroid angiogenesis. The maximum expression was observed at a VEGF concentration of 1 ng/ml, which is the same concentration at which VEGF is mitogenic to thyroid endothelial cells. Therefore, VEGF could have dual effects, both as a direct mitogen and to potentiate the actions of FGF-2. Similar experiments looking at the effects of TSP-1 on FGFR1 expression found no effect (data not shown). These results also show that thyroid endothelial cells respond in a different way to VEGF than other endothelial cell lines and indeed show the heterogeneity between endothelial cells lines (48). For example, the results of Yashima et al. (48) suggest that VEGF-induced intracellular signaling pathways vary depending on the origin of endothelial cells. Especially, Ras plays a dominant role in endothelial cells from capillary vessels, whereas protein kinase C does in endothelial cells of large vessels. These data suggest that the increases in VEGF seen during goitrogenesis may be acting in conjunction with the increased FGF-2 to initiate the angiogenic response.

In summary, we have isolated a human thyroid endothelial cell strain, confirmed by Tie-2 and factor VIII-related antigen expression and NO release in re-

sponse to VEGF. These cells respond to paracrine FGF-2, and VEGF, though a less potent mitogen, was able to increase FGFR1 expression. The cells also respond to the paracrine antiangiogenic factor TSP-1 and to angiostatin generated from plasminogen by the action of thyroid follicular cell-conditioned medium. This, and the observation that TSH and thyroid hormone had no apparent effect on thyroid endothelial cells, suggests that the angiogenesis observed during goitrogenesis in under the control of TSH-induced paracrine factors.

We thank Dr. Spencer Campbell for performing the plasminogen activator assays. We also thank Professor Asif Ahmed of the Dept. of Obstetrics and Gynecology, University of Birmingham, for the use of the Siemans 280 NO Analyzer and M. Grohman for technical assistance with the machine.

This work was supported by the University of Birmingham (V. A. Patel), the Wellcome Trust (M. C. Eggo), and the Medical Research Council (J. D. Ramsden).

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Am J Physiol Endocrinol Metab 297: E562, 2009;
doi:10.1152/ajpendo.zh1-5765-corr.2009.

Volume 284, January 2003

Patel VA, Logan A, Watkinson JC, Uz-Zaman S, Sheppard MC, Ramsden JD, Eggo MC. Isolation and characterization of human thyroid endothelial cells. *Am J Physiol Endocrinol Metab* 284: E168–E176, 2003. First published September 3, 2002; doi:10.1152/ajpendo.00096.2002; http://ajpendo.physiology.org/cgi/content/full/284/1/E168.

The European Collection of Cell Cultures (ECACC) has advised that multiplex analysis of the cell line deposited with them in 2006 and described by Patel et al. shows an identical match to that of the JEG3 cell line. ECACC used a new set of PCR (forensic) primers to detect this coidentity, which initial analyses by ECACC had not uncovered. The JEG3 cell line was derived from a human choriocarcinoma, and the cell-conditioned medium from this line was used to support the growth of the putative endothelial cell line. ECACC has withdrawn this cell line, known as TEC61, from distribution. No early-passage cells exist, so it is not possible to determine when cross-contamination occurred. Since the overall premise and conclusions of the paper can no longer be assured, the authors would like to retract the paper. We sincerely apologize to the Editorial Board and readership of the *American Journal of Physiology*.