Thyroid follicular cells secrete plasminogen activators and can form angiostatin from plasminogen

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Abstract
Angiostatin, a 38 kDa fragment of plasminogen, potently inhibits the growth of blood vessels. Angiostatin is generated from plasminogen by urokinase-type (uPA) and tissue-type (tPA) plasminogen activators in the presence of free sulphydryl donors. Angiogenesis inhibitors may be important in regulating angiogenesis in developing goitre. We have examined angiostatin formation in human primary thyrocyte cultures and a rat thyrocyte cell line (FRTL-5). We found that human thyroid cells in culture secrete plasminogen activators (both tPA and uPA) as well as matrix metalloproteinase 2 into the medium. When human thyrocyte conditioned medium was incubated with plasminogen (10 µg/ml) and N-acetylcysteine (100 µM) for 24 h, a 38 kDa fragment of plasminogen, which is consistent with angiostatin, was generated. The appearance of the 38 kDa fragment was increased by agents that increase cAMP (forskolin and 8 BrcAMP). FRTL-5 cells, which do not secrete uPA or tPA, did not generate angiostatin. Thyroid cells produce several angiogenic growth factors, and human thyrocyte conditioned medium stimulated growth of endothelial cells. When the conditioned medium was incubated with plasminogen and N-acetylcysteine, this stimulatory effect was lost, consistent with the production of a growth inhibitory factor. We conclude that thyroid cells can produce angiostatin from plasminogen in vitro, and this may play a role in vivo in limiting goitre size.


Introduction
Angiogenesis is the sprouting of new blood vessels from pre-existing vessels, and requires the recruitment of endothelial cells, mitosis, tube formation and re-constitution of functional vessels. It is essential for all postnatal tissue growth, and all tumour growth greater than 1–2 mm (Folkman 1972, Folkman & D’Amore 1996). There are both promoters and inhibitors of angiogenesis which maintain a tight control of blood vessel density, and this balance can be disturbed in diseases such as diabetic retinopathy and rheumatoid arthritis as well as in tumour growth and metastasis (Risau 1997). There is little angiogenesis in the adult vasculature except for wound healing and the menstrual cycle in the female reproductive tract, which makes development of anti-angiogenic oncologic agents to prevent neovascularisation of developing cancers a very promising therapeutic goal (Folkman 1995).

Angiostatin was identified by Folkman and colleagues (O’Reilly et al. 1994) and is a potent inhibitor of angiogenesis. Angiostatin has been shown to reduce angiogenesis in vitro and in vivo (Cao et al. 1996) and tumour growth in several animal models (O’Reilly et al. 1994, Tanaka et al. 1998). Angiostatin can be generated in vitro from plasminogen by both urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) (Gately et al. 1997). A free sulphhydryl donor (FSD) such as N-acetylcysteine, cysteine or reduced glutathione is also required. Angiostatin can also be generated from plasmin, in the presence of an FSD and in the absence of plasminogen activators, demonstrating that plasmin, itself a protease, is an essential intermediate that serves as both a substrate and an enzyme for angiostatin generation. This reaction requires the reduction of two disulphide bonds, Cys511-Cys535 and Cys461-Cys540, within kringle 5 of plasminogen. After the reduction of plasmin, proteolysis by serine proteases including plasmin and macrophage-derived metalloelastase produces the angiostatin fragment (Stathakis et al. 1997). This, under the correct conditions, can autolyse to a fragment of variable size, but the fragment containing kringle 1–3 of plasminogen is anti-angiogenic. Angiostatin is currently undergoing clinical trials as an anti-cancer agent.

Goitre formation is associated with enlargement of capillaries and mitosis of endothelial cells (Wollman et al. 1978) and in experimental models of iodine-deficient goitre endothelial proliferation occurs before thyroid...
epithelial cell growth (Wollman et al. 1978, Denef et al. 1981, Smids & Wollman 1983, Many et al. 1984). Many pro-angiogenic growth factors are produced by thyroid follicular cells, including insulin-like growth factor (Bachrach et al. 1988), vascular endothelial growth factor (Sato et al. 1995), fibroblast growth factor (FGF) (Thompson et al. 1998) and angiopoietin–1 (Cocks et al. 2000, Ramsden et al. 2001), and the conditioned medium of thyroid cells stimulates endothelial cell growth (Goodman & Rone 1987). An inhibitor of angiogenesis, thrombospordin, is associated with the extracellular matrix in the thyroid, and its release is reduced by thyrotrophin (TSH) stimulation (Patel et al. 1996). Although angiostatin was identified as a regulator of angiogenesis in malignant disease, it has also been generated by macrophages in models of inflammatory disease (Falcone et al. 1998), and may play a role in normal tissue growth. However, the role of angiogenesis inhibitors is not fully established in non-malignant tissue growth, such as goitre formation, or in the regulatory factors that limit goitre formation in disease such as Graves’ disease. We believe that local inhibitors of angiogenesis play an important role in limiting goitre size in response to elevated thyrotrophin.

We hypothesised that the follicular cells in the thyroid can generate angiostatin by the secretion of proteases that degrade plasminogen, and that this would inhibit endothelial cell growth.

Materials and Methods

Cell culture

Human thyroid follicular cells were prepared from surgical specimens as described (Eggo et al. 1996). In brief, thyroid tissue (normal and multinodular goitre) was digested using 0·2% collagenase. Follicles were plated in the modified F12M medium described by Ambesi-Impiombato et al. (1980), supplemented with TSH (300 mU/l), insulin (100 µg/l), penicillin (105 U/l), streptomycin (100 mg/l) and 0·2% collagenase. Follicles were plated in the modified F12M medium supplemented with TSH (300 mU/l), insulin (100 µg/l), penicillin (105 U/l), streptomycin (100 mg/l), 2·4 × 105 U/l nystatin, 1·0 µg/1 FGF2 and 17·5 U/ml heparin.

Western blots

Proteins in the cell lysates were heated at 100 °C for 5 min in 1% sodium dodecyl sulphate (SDS). The protein concentration was assayed using BioRad Protein Assay (BioRad Laboratories, Hercules, CA, USA) and 100 µg protein were reduced by boiling in 2% 2-mercaptoethanol. Secreted proteins were precipitated using 3 volumes absolute ethanol at −20 °C for 2 h. Following recovery by centrifugation, the precipitated proteins were resuspended in sample buffer containing 1% SDS, 2% 2-mercaptoethanol, 20% glycerol and 62·5 mM Tris–HCl pH 6·8, bromophenol blue and boiled for 5 min. Proteins were analysed by electrophoresis on denaturing 10% SDS-polyacrylamide gels (PAG) with 5% stacking gel. The proteins were transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). Membranes were blocked in 10% milk in TBST (10 mM Tris–HCl, 100 mM NaCl, 0·1% Tween–20, pH 7·5) for 1 h and incubated in primary antibody at optimal dilution in 5% milk in TBST. Primary antibodies used were polyclonal anti-human plasminogen, anti-human tPA and anti-uPA, all from Binding Site, Birmingham, UK. The membrane was washed 3 times for 15 min in TBST and placed in anti-sheep/goat immunoglobulin conjugated to horseradish peroxidase secondary antibody (Binding Site) for 1 h. Membranes were developed using enhanced chemiluminescence (ECL, Amersham International, Amersham, Bucks, UK) and exposed to X-ray film (Kodak Biomax MR, Rochester, NY, USA) with intensifying screens. Membranes were stripped with 62·5 mM Tris, 2% SDS and 1·5% 2-mercaptoethanol pH 6·8 at 60 °C for 30 min before reprobing.

Endothelial cell growth assays

Thyroid cells, 10 days post isolation, were cultured in serum-free medium which was collected after 72 h and centrifuged to remove all cells. The supernatant was incubated with or without 100 µM N-acetylcysteine and 10 µg/ml plasminogen (Sigma-Aldrich) for 24 h at 37 °C. Medium incubated in the presence and absence of plasminogen and N-acetylcysteine was used as control medium. In some experiments cysteine was substituted for


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N-acetylcysteine as described by Gately et al. (1997). Following incubation the medium was added to HUVE and hMVE cells (cultured to 70% confluence) mixed with an equal volume of endothelial cell medium. The endothelial cell medium was made as described previously except the FCS was heat inactivated at 55°C or 1 h to denature proteases and plasminogen. The medium was supplemented with [methyl-3H]thymidine at a final concentration of 0.5 µCi/well.

The endothelial cells were incubated for 24 h at 37°C and the medium removed, cells were washed in HBSS, and the cells were precipitated with 6% trichloroacetic acid (TCA) at 4°C. The cell layer was washed with 6% TCA, dissolved in 0.1 M NaOH and radioactivity was determined by liquid scintillation counting.

Statistical analysis
The results were analysed using INSTAT (Graphpad Software, San Diego, CA, USA). The mean d.p.m. and standard deviations were calculated. Differences were analysed by ANOVA using the Tukey–Kramer multiple comparisons post test. Comparisons were considered significant when P<0.05.

Results
Expression of uPA and tPA by thyroid follicular cells
The secreted proteins in human thyroid cell conditioned medium from cells treated with TSH, EGF, and the protein kinase C (PKC) activator, 12-O-tetradecanoylphorbol 13-acetate (TPA), were analysed by Western blotting. EGF and TPA are known to increase PA production in other cell types. PKC activation is commonly associated with growth factor signalling. Membranes were probed with polyclonal antisera to uPA, and, without stripping, subsequently probed for tPA. Both proteins were present in the medium (Fig. 1). Although in this experiment TSH increased tPA expression, further experiments using TSH dose responses did not confirm that TSH increased either uPA or tPA expression consistently. EGF and activation of PKC with TPA increased the expression of both uPA and tPA. The cell layer contained little or no detectable PAs (data not shown).

Expression of matrix metalloproteinase-2 (MMP-2) by thyroid follicular cells
Since MMP-2 can also generate angiostatin from plasminogen (Dong et al. 1997, 1998), we examined the expression of MMP-2 in conditioned medium from human follicular cells treated with increasing concentrations of TSH. MMP-2 was present in the medium but was not increased by TSH (Fig. 2).

Degradation of plasminogen and formation of a 38 kDa fragment by thyrocyte conditioned medium
The cell-free conditioned medium (CFCM) of thyroid follicular cells stimulated with 0.3 mU/ml TSH was incubated with 100 µM N-acetylcysteine and 10 µg/ml plasminogen for varying times, analysed by Western blotting, and the membrane probed with a polyclonal antibody to plasminogen. There was increasing degradation of plasminogen over time by conditioned medium, and none by control medium that had not been exposed to thyroid cells as shown in Fig. 3. By 24 h full length plasminogen was nearly completely degraded and by 48 h no plasminogen was detectable (Fig. 3). After 24 h a 38-kDa fragment of plasminogen was detected which corresponds with the size of angiostatin (O’Reilly et al. 1994). This, too, was degraded by 48 h.

Regulation of angiostatin production by agents that elevate intracellular cAMP
Most of the effects of thyrotrophin are transduced through elevations in cAMP. To determine the role of this pathway
in the regulation of angiostatin formation we used forskolin to activate adenylate cyclase and 8 BrcAMP to mimic the effects of cAMP in human follicular cells. The CFCM of thyroid follicular cells grown in medium without TSH (lane 2) or supplemented with 100 µM forskolin (lane 3) or 5 mM 8 BrcAMP (lane 4) was incubated with plasminogen and N-acetylcysteine for 24 h. Proteins were analysed by Western blotting and the membrane probed with a polyclonal antibody to plasminogen (Fig. 4). The plasminogen control (lane 1) was not degraded, while CFCM degrades plasminogen to plasmin and also to a 38 kDa fragment. The formation of this fragment was increased if the cells were stimulated by agents that elevate intracellular cAMP.

Thyroid follicular cells produce angiostatin that inhibits endothelial cells growth

To show that the fragment of plasminogen of 38 kDa possesses the growth inhibitory properties of angiostatin, we examined the biological effect of the CFCM of human thyroid cells on endothelial growth. The medium of thyroid cells is stimulatory to endothelial cells, and thyroid cells secrete a number of endothelial growth factors (Goodman & Rone 1987, Greil et al. 1989, Ramsden 2000, Ramsden et al. 2001). HUVE cells were grown in a 50:50 mix of their medium and a test medium for 72 h. The test media were unconditioned medium incubated with or without plasminogen or the CFCM of thyroid cells after 24-h incubation with or without plasminogen. Plasminogen alone did not promote or inhibit growth compared with control. In the hMVE cells, thyroid cell conditioned medium stimulated a 1.5-fold increase in [methyl-3H]thymidine uptake after 24 h which was again abolished by thyroid CFCM incubated with plasminogen (Fig. 5b).

The growth of FRTL-5 cells is not inhibited by angiostatin

To determine whether the factors formed by proteolytic degradation of plasminogen are specific to endothelial cells we also examined their effects on the growth of FRTL-5 cells. FRTL-5 cells were grown in conditioned medium of human thyroid cells and CFCM was incubated with plasminogen for 24 h as described for the experiments with endothelial cells. There was no difference in the growth assayed by [methyl-3H]thymidine uptake (data not available).
shown), showing that the growth inhibitory effect of angiostatin on endothelial cells is specific and does not affect epithelial cells.

**Discussion**

We have shown that plasminogen activators, uPA and tPA, are produced by human thyroid cells in culture. We found that both of the PAs were identified in the conditioned medium, but were not detectable in the cell layer, suggesting that in thyroid cells they are predominantly secreted rather than membrane bound. This is similar to previous findings in sheep thyrocytes (Eggo et al. 1987). We have also shown that the conditioned medium of human thyroid cells degrades plasminogen, and when plasminogen is degraded by secreted thyrocyte proteases, a fragment of 38 kDa is detected. Medium containing this fragment is growth inhibitory to endothelial cells but not to FRTL-5 cells, consistent with the endothelial-specific activity of angiostatin. We also found that FRTL-5 cells, which have previously been shown not to secrete PAs (Eggo et al. 1987, Cassano et al. 1989), do not degrade plasminogen in culture, or generate angiostatin.

Matrix metalloproteinases have also been reported to generate angiostatin from plasminogen by O’Reilly et al. (1999), who first described angiostatin. Plasminogen degradation to angiostatin can thus occur by several proteolytic pathways. In our studies the expression of PAs and MMP-2 was not increased by TSH. However both uPA and MMP-2 are secreted as latent proforms requiring activation, and TSH may regulate this process. PA activity is similarly regulated by other proteins including PA inhibitors -1 and -2 and conceivably TSH may act to modulate their synthesis rather than by regulating PA expression. Consistent with this interpretation, we found increased angiostatin generation by medium from thyroid cells treated with agents that elevate intracellular cAMP. In sheep thyroid cells we found increased PA activity with TSH (Mak et al. 1984) but did not examine PA protein expression by Western blotting. Both uPA and PA inhibitor-1 have been identified in human thyroids by immunohistochemistry, and were found to be increased in both papillary and follicular tumours (Ito et al. 1996), although no effects on prognosis or clinico-pathological staging were identified. In other tumours uPA and tPA are postulated to increase tumour growth and metastasis due to their ability to degrade the extracellular matrix (Smit et al. 1999). However, no firm evidence for this process in thyroid cancer exists, and in metastatic thyroid tumour uPA-inhibitor is increased (Chen et al. 2001).

Other controls on angiostatin production could be due to the local concentrations of plasminogen or uPA. Plasminogen is known to bind to endothelial and other cell types with moderate affinity (120–340 nM) and high capacity (4–14 × 10⁵ molecules per cell (Van Hinsbergh et al. 1997)). Similarly uPA has a specific receptor which is found on endothelial cells and thyroid cells and is upregulated by its ligand (Van Hinsbergh et al. 1997, Montuori et al. 2000). Regulation of these receptors or the interactions between the ligands bound to these receptors is conceivable. PAs are also synthesised by endothelial cells and their contribution to angiostatin production as well as matrix invasion should be considered (Van Hinsbergh et al. 1997).

Regulation of thyroid angiogenesis is a balance between promoters and inhibitors present within the thyroid gland. During goitre formation promoters predominate as there is a rapid angiogenesis. However, in the majority of goitres
there is a limitation of goitre size, not directly related to biochemical thyroid status, and it may be that at this stage of goitrogenesis there is a switch in the balance of angiogenic factors from pro- to antiangiogenic. Inhibitors of angiogenesis such as angiotatin, thrombospondin and angiopoietin-2 are likely to be important at this stage. Although the \textit{in vivo} role of angiotatin in the regulation of non-malignant tissue growth is unknown, our studies support a potential role, as both plasminogen and plasminogen activators are present in the thyroid.

Our study suggests that thyroid cells can generate an inhibitor of angiogenesis, angiotatin. This compound may reduce growth associated with hyperplasia and neoplasia in the thyroid, and could be a potential treatment for differentiated thyroid cancer.

Acknowledgements

The financial support of the University of Birmingham (S Y, E D), the Medical Research Council (J D R) and the Wellcome Trust (M C E) is gratefully acknowledged.

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Received 29 December 2001
Accepted 28 February 2002