COMMENTARY

Angiogenesis in the thyroid gland

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Abstract

Angiogenesis is the mechanism of blood vessel formation after the first few days of embryogenesis, and is essential for all tissue growth. In adults, angiogenesis occurs in the thyroid during disease processes including goitre, Graves' disease, thyroiditis and cancer. The molecular mechanisms controlling angiogenesis are becoming clearer, and therapy targeting these processes is coming closer to clinical fruition. Both promoters and inhibitors of angiogenesis

have been identified in the thyroid, including vascular endothelial growth factor (VEGF), fibroblast growth factor, and thrombospondin. This commentary will review the understanding of the control of angiogenesis within the context of the thyroid gland, and review the pre-eminent role of VEGF as the angiogenic signal from the follicular cells to the endothelial cells.

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Introduction

Angiogenesis is the sprouting of new blood vessels from pre-existing capillaries, and requires the multiplication of endothelial cells, their migration, remodelling of the extracellular matrix, tube formation and recruitment of surrounding structures to maintain the newly formed vessels. Before a functional network is formed, anastomoses must be made with other new or pre-existing vessels, and the basement membrane must be established (Risau 1997). This process is tightly controlled, and occurs rarely in the adult vasculature except for wound healing, the menstrual cycle in the female reproductive tract, and pathological conditions including diabetic retinopathy and tumour formation (Folkman 1995). Tumour formation requires the development of a blood supply to allow growth larger than 2 mm in diameter, and thus inhibition of angiogenesis could prevent tumour growth and metastasis (Folkman 1971). Very little angiogenesis occurs in normal adult blood vessels, which makes the development of treatments to prevent vessel growth a very promising therapeutic goal. It has been recognised recently that promotion of blood vessel growth can ameliorate the severity of occlusive vascular disease, such as coronary and peripheral ischaemic disease (Henry 1999).

Increased vascularity in the thyroid can occur in hyperplastic goitre, Graves' disease and cancer, and may be associated with a vascular hum because of increased blood flow. In cancers of the thyroid, microvessel density has been shown to correlate with disease-free survival in papillary carcinoma of the thyroid (Ishiwata *et al.* 1998)

and intrathyroid tumour spread in follicular carcinoma (Segal *et al.* 1996).

In experimental induction of goitre by low iodine and thiouracil in rats, Wollman *et al.* (1978) showed that the capillaries within the thyroid clearly enlarged within 3 days of treatment, and by 20 days they almost completely surrounded the follicles with a continuous endothelial sheet. There was both fusion of capillaries and mitosis of endothelial cells. There was no change in blood vessel morphology or number in nearby extrathyroidal tissue, including the parathyroids. In this model, endothelial cell proliferation occurs before thyroid epithelial cell growth (Wollman *et al.* 1978, Denef *et al.* 1981, Smeds & Wollman 1983, Many *et al.* 1984), and Wollman and colleagues suggested that the mechanism of endothelium growth stimulation might be the action of substances produced by neighbouring epithelial cells. Subsequently, Goodman & Rone (1987) showed that follicular cells *in vitro* release into the conditioned medium a growth factor that causes endothelial cell growth and migration, and that this was a heparin-binding protein.

The thyroid is an excellent model for understanding co-ordinated angiogenesis, because during goitre formation there is growth of follicular and endothelial cells, and it is probable that this is regulated by the release from follicular cells of cytokines in response to low concentrations of tri-iodothyronine and high concentrations of thyroid-stimulating hormone (TSH). Numerous factors have been identified that modulate angiogenesis; these include both promoters and inhibitors, which interact to control organised development of the new blood vessels.

Table 1 Angiogenic growth factors

Promoters	Inhibitors
Vascular endothelial growth factor	Thrombospondin
Fibroblast growth factor	Angiostatin
Angiopoietins-1 and -2	Endostatin
Angiogenin	
Hepatocyte growth factor	
Nitric oxide	
Interleukin-8	
Platelet-derived growth factor	
Placental growth factor	
Proliferin	
Endothelin	

The function of some of these factors is unknown in the thyroid, although it is reasonable to speculate that many of them will play a role. These growth factors are summarised in Table 1.

Vascular endothelial growth factor

The principal specific promoter of endothelial cell growth and migration is vascular endothelial growth factor (VEGF), which is a secreted homodimeric glycoprotein of 46 kDa. It is chemoattractant and mitogenic for endothelial cells, and *in vivo* produces angiogenesis and increases vascular permeability (Ferrara *et al.* 1992). There are several different members of the VEGF family – VEGF-A, -B, -C and -D (Carmeliet & Collen 1997, Achen *et al.* 1998). All are transcribed from a single gene, then processed into several isoforms that have different tissue expression, but which bind to tyrosine kinase cell membrane receptors – VEGFR-1, -2 and -3 (also called Flt-1, Flk/KDR, and Flt-4 respectively). VEGFR-1 and -2 are principally expressed on endothelial cells. The predominant isoform is VEGF-A, which has four main configurations: VEGF-A¹²⁰, VEGF-A¹⁶⁵, VEGF-A¹⁸⁹ and VEGF-A²⁰⁶. Another homologue, 46% identical to VEGF-A, is placental growth factor (PlGF), which was identified in the placenta, but is also expressed in heart, lung and thyroid (Maglione *et al.* 1991, Viglietto *et al.* 1995, 1997). It binds to VEGFR-1 and can form heterodimers with VEGF-A. It can significantly potentiate the effect of VEGF-A *in vitro* and *in vivo*. The binding of the VEGF isoforms to the receptors is summarised in Fig. 1.

There is accumulating evidence that VEGF and its receptors are important in the thyroid in Graves’ disease (Viglietto *et al.* 1997), thyroiditis (Klein *et al.* 1999), goitre (Wang *et al.* 1998) and cancer (Soh *et al.* 1996, 1997).

In vitro human thyroid follicular cells grown in culture can synthesise and secrete VEGF-A, and the basal level of mRNA expression can be increased by factors signalling

Figure 1 Binding of VEGF isoforms to the receptors.

through the cAMP and protein kinase C pathways, including TSH, dibutyryl cAMP and Graves’ disease serum (Sato *et al.* 1995). *In vivo*, using a model of induced goitre in thiouracil-fed rats, VEGFR-1 and -2 mRNA expression increases during goitre formation, and this occurs subsequent to the increase in plasma TSH, and in parallel with thyroid capillary proliferation. In Graves’ disease there is enhanced expression of mRNA of PlGF and VEGF in addition to VEGFR-1 and -2 (Viglietto *et al.* 1997) as measured by northern blot hybridisation. There is also an increased serum VEGF concentration compared with that in healthy individuals and, using colour flow Doppler ultrasonography, there is a significant correlation between serum VEGF concentration and the ratio of intrathyroidal vascular area to thyroid area, in a subgroup of patients with Graves’ disease and goitre. This increased vascularity, in addition to the serum VEGF concentration, was reduced after treatment with antithyroid drugs (Itaka *et al.* 1998).

VEGF also seems to be important in thyroid tumours. Thyroid cancer cell lines in culture secrete more VEGF than normal thyrocytes, and in these cell lines TSH increases the secretion of VEGF measured by ELISA (Soh *et al.* 1996, 1997). Interestingly, in a cell line derived from Hürthle cell cancer (XTC-1) there was high basal secretion of VEGF that was not increased by TSH, suggesting that VEGF secretion may be constitutively activated in some thyroid cancers (Soh *et al.* 1997). The expression of VEGF *in vitro* has been shown to correlate with *in vivo* aggressiveness of the tumours (Viglietto *et al.* 1995), with anaplastic tumours having the greatest level of expression of VEGF. Constitutively overexpressing the VEGF gene in a thyroid tumour cell line does not alter *in vitro* growth, but increases vascularity and reduces tumour latency when the tumour cell line is injected subcutaneously in nude mice (Belletti *et al.* 1999). Immunostaining of thyroid tumours shows co-localisation of increased Ki67 and VEGF staining, and this may be because areas of tumour with rapid cell division are increasing VEGF secretion to promote angiogenesis (Klein *et al.* 1999). The same study also showed greater

immunostaining for VEGF in tumours that had metastasised, compared with those that had not.

It is clear that VEGF is produced by follicular cells – probably in response to TSH, among other signals – and that this cytokine exerts effects on intrathyroidal endothelial cells via the VEGFR-1 and -2. VEGF receptors have not been identified on the follicular cells, although there is an intriguing report that the VEGFR-1 mRNA has been found by RT-PCR in Fisher rat thyroid (FRTL-5) cells (Wang *et al.* 1998); it is also not known if VEGF has an autocrine effect on follicular cells. VEGF seems to be a significant component of the regulation of angiogenesis within the thyroid and alteration in intrathyroid expression of VEGF is seen in many thyroid pathologies.

Fibroblast growth factor (FGF)

The FGF gene family consists of 13 members, which are produced by a wide range of cell types (for review see Bikfalvi *et al.* 1997). FGF-1 (acidic) and FGF-2 (basic) are unusual, as they lack a signal peptide necessary for the classical secretory pathway. They have high affinity for heparan sulphate glycosaminoglycans in the extracellular matrix and, although the mechanism of FGF release is not understood, there are significant amounts of FGF associated with the extracellular matrix. FGF-2 has many functions, including proliferation and chemotaxis of several cell types, including fibroblasts, endothelial cells and smooth muscle cells (Baird & Bohlen 1990), and it is a potent stimulator of angiogenesis. Another mechanism whereby FGF may regulate angiogenesis is the secretion of binding proteins into the extracellular matrix. These binding proteins bind to and displace FGF from their bonds to heparan sulphate proteoglycans, and the displaced and soluble FGF-2 molecules can bind to receptors, initiating biological effects (Czubayko *et al.* 1997).

Human follicular cells express both FGF-1 and -2 in addition to the receptor FGFR-1, and expression of these factors is increased in multinodular goitre (Thompson *et al.* 1998). Moreover, FGF-1 is expressed by pig and sheep thyroid cells in culture, and is a potent goitrogen in rats, increasing thyroid weight by 43% after 6 days of treatment (Bidey *et al.* 1999). It is plausible that FGF alone may be acting as an angiogenic factor in the thyroid, with direct effects on both endothelial and follicular cell growth. However, in other tissues there is interdependence of FGF and VEGF. FGF-2 induces the expression of VEGF by endothelial cells, and the mitogenic effects of FGF-2 on endothelial cells can be blocked with anti-VEGF antibodies *in vitro* and *in vivo* (Seghezzi *et al.* 1998). Conversely, VEGF increases the mitogenic effects of FGF-2 on vascular smooth muscle cells in a model of rat carotid arteries injured by balloon inflation (Couper *et al.* 1997). It appears that there are complex relationships in the regu-

lation of VEGF and FGF, and accordingly it is likely that there are interactions between the two cytokines within the thyroid, although it requires further investigation to confirm this in goitrogenesis.

Matrix metalloproteinases (MMPs)

Degradation of the extracellular matrix is essential for the formation of new blood vessels to allow in-growth of the endothelial cells, and this requires MMPs, some of which have been identified in the thyroid. Their expression can be regulated at the transcriptional level by cytokines, including VEGF, and post-translationally by interaction with other MMPs and tissue matrix metalloproteinase inhibitors (TIMPs) (Wang & Keiser 1998). Normal thyrocytes in culture express mRNA for MMP-2, whereas thyroid anaplastic carcinoma cell lines express MMP-1, -2 and -9 and TIMP-1, -2 and -3 mRNA *in vitro* (Aust *et al.* 1997). Interestingly, the thyroid cell type that secretes the greatest amounts of MMPs are fibroblasts, and these are stimulated by phorbol esters to increase secretion of a number of MMPs, including MMP-2 and -9 (Hofmann *et al.* 1998). This would concur with a previous study that found expression of MMP-2 mRNA predominantly restricted to fibroblasts in thyroid tumours (Zedenius *et al.* 1996). It is known neither by what signal thyroid tumours might stimulate MMP release in surrounding fibroblasts, nor the role of follicular cells in regulating MMPs.

Inhibitors of angiogenesis

Thrombospondin-1 (TSP-1) is an inhibitor of endothelial cell growth associated with the extracellular matrix. It is present in the thyroid, where it is found in the stroma, and it is secreted from the apical borders of follicular cells (Prabakaran *et al.* 1993). Its release is inhibited by TSH, and concentrations of stromal thrombospondin decrease after 2 weeks of goitre induction, concurrently with angiogenesis occurring (Patel *et al.* 1996). In human thyroid tumours there is a reduction in thrombospondin mRNA and protein, and this is greatest in follicular and undifferentiated cancer (Bunone *et al.* 1999). Conceivably, during thyroid growth there are reduced stromal concentrations of TSP-1, possibly in response to increased TSH, which would alter the balance of pro- and anti-angiogenic factors within the thyroid.

Angiostatin is a fragment of plasminogen with anti-angiogenic properties (O'Reilly *et al.* 1994). It is generated by urinary-type (uPA) and tissue-type (tPA) plasminogen activator in the presence of free sulphhydryl donors (Gately *et al.* 1997). Angiostatin potently inhibits the growth of blood vessels, and prevents growth of both primary tumours and metastases (Sim *et al.* 1997). Both uPA and tPA are present within the thyroid, and are increased by

TSH *in vitro* (Mak *et al.* 1984). This could lead to an increase in angiostatin formation, which theoretically may have a role in negatively regulating blood vessel formation in response to TSH, and limiting goitre size.

Angiopoietins

The angiopoietins are 70 kDa secreted proteins that are essential for angiogenesis. The family consists of angiopoietins-1 (Ang-1) and -2 (Ang-2), which act via a membrane tyrosine kinase receptor, Tie-2 (Davis *et al.* 1996, Maisonpierre *et al.* 1997). Ang-1 phosphorylates Tie-2, promoting endothelial cell migration, tube formation, and the stabilisation of new capillaries. Ang-2 binds to Tie-2 with similar affinity, but does not phosphorylate the receptor; it acts as a physiological antagonist to Ang-1. The function of Ang-2, however, seems to be more than simply opposing Ang-1, and it seems to regulate the response of endothelial cells to other angiogenic stimuli. To date there has been a single report that there is increased mRNA for Ang-2 and Tie-2 in thyroid neoplasia measured with RT-PCR, and this association is particularly strongly related to tumour size and progression from a pre-vascular to a vascular phase (Bunone *et al.* 1999).

Vasoactive compounds

Endothelin-1 (ET-1) is a potent vasoactive peptide that modulates vasomotor tone and endothelial cell proliferation. ET-1 and its receptors, ETs-A and -B, are expressed in the thyroid and increased during goitre formation (Colin *et al.* 1995). ET-1 may increase angiogenesis directly or via VEGF, as ET-1 can stimulate VEGF production (Pedram *et al.* 1997). Alternatively, the vasoactive actions of ET-1 may directly contribute to the alterations in thyroid blood flow seen in disease. Nitric oxide (NO) is another potent vasoactive compound synthesised by NO synthase (NOS) enzymes in the thyroid that also may have angiogenic functions. The vascular expansion during experimental goitre formation is reduced by antagonists of NOS, although total goitre size is not affected (Colin *et al.* 1995). The relationships of these vasoactive compounds to overall regulation of blood vessel growth has not been fully elucidated.

Future research objectives

The relative contribution of the different angiogenic factors within the thyroid remains to be established, especially their relationship with thyroid disease. Before treatments exploiting angiogenic strategies are used, it would be relevant to determine whether the most im-

portant signal for angiogenesis in the thyroid is the removal of anti-angiogenic factors such as thrombospondin, or the expression of pro-angiogenic cytokines such as VEGF. It may then be possible to modify the angiogenic climate within the thyroid to prevent goitre or tumours.

Summary

There are many positive and negative regulators of angiogenesis, and a plethora of new cytokines have recently been identified and implicated in the regulation of blood vessel growth. However, on current understanding, VEGF is a central factor in angiogenesis in the thyroid gland, as indicated by the alterations in VEGF concentrations in many pathological conditions of the thyroid. Many other factors are involved, although some of these may act through regulation of VEGF expression in follicular cells. TSH is an important regulator of VEGF, although the role of factors important in other tissues, such as hypoxia, is not known in the thyroid. The thyroid is an excellent model for the integrated control of angiogenesis, because of the vascularity of the thyroid gland, and its capacity to increase its blood flow in disease. Further understanding of these processes may allow clinical treatments for thyroid disease, including goitre and cancer.

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