Review

VEGF in physiological process and thyroid disease

Le VEGF en physiologie et pathologie thyroïdienne

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Abstract

Angiogenesis is a physiological process involving the growth of new vessels from pre-existing vasculature. Vascular endothelial growth factor (VEGF) is an important regulator of both benign and malignant disease processes in the thyroid gland. The VEGF family includes seven members respectively named VEGF-A, also known as VPF (vascular permeability factor), VEGF-B, VEGF-C, VEGF-D, all described in mammals, VEGF-E (found in Parapoxviridae), VEGF-F (also known as svVEGF for snake venom VEGF, found in viper venom) and PlGF (placental growth factor). Thyrocytes are able to synthesize and secrete VEGF. VEGF-A is implicated in tumour growth and metastasis via blood vessels while VEGF-C and VEGF-D, involved in lymphangiogenesis, favour metastasis to the cervical lymph nodes in papillary thyroid carcinomas. High levels of VEGF expression in thyroid tumour cells may correlate with a poorer outcome in papillary thyroid carcinomas. Because of its important role in malignant angiogenesis, VEGF is the preferential target of a new variety of therapeutic agents called angiogenesis inhibitors.

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1. Introduction

Angiogenesis corresponds to the generation of new vessels from the pre-existing vasculature, as opposed to vasculogen-
Angiogenic factors belong to different classes of molecules including in particular the cytokines, growth factors, prostaglandins and proteolytic enzymes [40,67,95]. These factors act through receptors, the majority of which belong to the receptor tyrosine kinase family, which plays an integral role. Angiogenesis can also be induced by physical or physicochemical stimuli [38]. VEGF (vascular endothelial growth factor) plays a pivotal role in angiogenesis. This 40 kD glycoprotein, which is usually homodimeric, is a growth factor from the PDGF family (platelet-derived growth factor). It is secreted by many cell types and has been detected in the incubation medium of normal pituitary folliculostellar cells (PFSC) [32] as well as in many transformed cells.

The VEGF family includes seven members respectively named VEGF-A, also known as VPF (vascular permeability factor), VEGF-B, VEGF-C, VEGF-D, all described in mammals, VEGF-E (found in paraposviridae), VEGF-F (also called SV-VEGF, for snake venom VEGF, found in viper venom) and PIGF (placental growth factor). These variants share a common sequence containing a characteristic, conserved distribution of eight cysteine residues involved in intra- and intercatenary disulfide bridges. They exert their specific functions by binding to three different receptors from the receptor tyrosine kinase family: VEGFR-1 (also known as Flt-1 for Fms-like tyrosine kinase-1), VEGFR-2 (also called Flk-1 for fetal liver kinase-1 and in humans known as KDR for kinase insert domain-containing receptor), and VEGFR-3 (also called Flt-4 for Fms-like tyrosine kinase-4). Some of these factors also bind to different non-specific receptors including the semaphorin receptors Nrp-1 (neuropilin-1), and Nrp-2 (neuropilin-2) or to HSPGs (heparan sulfate proteoglycans).

2. The different VEGF isoforms and their receptors

2.1. VEGF-A

Nine different VEGF-A isoforms generated by alternative splicing of the same gene have been identified: VEGF121, VEGF145, VEGF148, VEGF162, VEGF165, VEGF165b, VEGF183, VEGF189 and VEGF206. The gene contains eight exons separated by seven introns and is located on chromosome 6p21.3. The human isoforms are VEGF121, VEGF145, VEGF165 (the most abundantly secreted isoform), VEGF189 and VEGF206. Certain properties are common to all isoforms while others are specific to each, resulting in selectivity for different receptors. In most cases the isoforms act as activators except for VEGF165b, which has an inhibitory function.

VEGF is produced by macrophages, activated T lymphocytes and even by endothelial cells themselves, as well as by a wide variety of normal and pathological tissues. Some isoforms such as VEGF121, VEGF165, and VEGF189 are preferentially secreted while others, including VEGF183, VEGF189 and VEGF206 are sequestered in the extracellular matrix from which they are released through the action of heparin or heparinases, or more rapidly after cleavage of the C-terminal end by plasmin or urokinase. VEGF-A was originally named VPF due to its ability to increase vascular permeability in skin capillaries and to stimulate the production of ascites fluid. It also induces the proliferation, tubular formation, branching and migration of EC. It prolongs cell survival by inducing the expression of anti-apoptotic proteins like Bcl-2 and A1 in EC. It stimulates vasodilation through induction of endothelial NO synthase (eNOS) and NO production.

Biological activity has also been demonstrated in other cell types including a mitogenic effect in retinal pigment epithelium, Schwann cells and smooth muscle fibers and a protective effect on anoxic motor neurons and in amyotrophic lateral sclerosis (ALS). Hematologic effects have also been described: production of leukocyte colonies, induction of monocyte chemotaxis and coagulation activation.

2.2. VEGF-B

The VEGF-B gene comprises eight exons and six introns and localizes to chromosome 11q13. The human gene codes for at least two isoforms: VEGF-B167, with a wide tissue distribution, particularly in skeletal and cardiac muscle and brown adipose tissue, and VEGF-B186 with a less extensive tissue distribution. VEGF-B binds VEGFR-1 and Nrp-1 and can form heterodimers with VEGF-A. The in vivo role of VEGF-B is still obscure but it is known that hypoxia does not induce its expression. Homozygous VEGF-B knockout mice were found to have smaller hearts and impaired recovery following myocardial ischemia, suggesting a role for VEGF-B in coronary angiogenesis.

2.3. VEGF-C

The VEGF-C gene maps to chromosome 4q34 and contains seven exons. The gene encodes a precursor protein that undergoes proteolytic maturation via extracellular proteases to the activated form, a heterodimer displaying high affinity binding to VEGFR-2 and VEGFR-3. It is also a ligand of Nrp-2. Tissue distribution includes the thyroid gland, heart, ovaries, small intestine and placenta. VEGF-C induces EC proliferation, migration and survival. Its main role appears to be as an activator of lymphangiogenesis, mediated through VEGFR-3 signal transduction. Homozygous VEGF-C knockout mice died in utero with an absence of lymphatic vessels and severe oedema. Conversely, transgenic mice overexpressing VEGF-C had lymphatic hyperplasia.

2.4. VEGF-D

This glycoprotein displays 48% homology with VEGF-C. It undergoes the same proteolytic maturation as the latter and binds to human VEGFR-2 and VEGFR-3 (and only VEGFR-3 in mice). The gene is located at Xp22.31. VEGF-D disruption has little effect on intrauterine life. It is thought to play a role in
angiogenesis and lymphangiogenesis during tumour growth, thereby promoting metastatic dissemination.

2.5. VEGF-E

Identified in the parapoxvirus Orf virus, VEGF-E may be partly responsible for the extensive capillary dilatation which is a characteristic histologic feature observed in skin lesions caused by these infections. All VEGF-E variants bind to VEGFR-2 and stimulate EC proliferation and vascular permeability. Some variants can also bind to Nrp-1.

2.6. VEGF-F or svVEGF

Recently discovered in snake venom, the two VEGF-F isoforms (vammin and VR-1) share 50% of the primary structure of VEGF165. VR-1 binds with high affinity to VEGFR-1 and with low affinity to VEGFR-2, while vammin shows high affinity binding to VEGFR-2, but neither binds to VEGFR-3 or Nrp-1. These svVEGF variants are thought to act as toxins in snake bite envenoming, although the underlying mechanisms have yet to be elucidated.

2.7. PlGF

PlGF was first identified in 1991 in human placenta, but it is also expressed in heart and lung. The gene, located on chromosome 14q24, contains seven exons. Alternative splicing generated four isoforms: PlGF-1 (PlGF131), PlGF-2 (PlGF152), PlGF-3 (PlGF203) and PlGF-4 (PlGF224). Since PlGF has a crystalline structure similar to that of VEGF-A, it can bind to the VEGFR-1 receptor. However it only binds VEGFR-2 in the form of a PlGF/VEGF heterodimer. PlGF-2 also binds to the Nrp-1 and Nrp-2 receptors. Even-numbered isoforms have a heparin-binding domain. While disruption of the gene does not interfere with intrauterine angiogenesis in mice, it does affect angiogenesis and vascular permeability during inflammation, ischemia, neoplasia and wound healing, thus underscoring the pivotal role of VEGFR-1 signalling pathways in these processes.

The different VEGF variants described above exert their activity by binding to receptor tyrosine kinases from the same subclass as the PDGF and FGF (fibroblast growth factor) receptors. These receptors were originally identified in EC. VEGFs can also bind to coreceptors, defined as molecules capable of binding VEGF, but devoid of coupled catalytic activity, such as heparan sulfate proteoglycans (HSPGs) and neuropilins. The VEGFR-1, -2 and -3 receptors belong to the receptor tyrosine kinase family.

2.8. VEGFR-1

VEGFR-1 is a high affinity receptor for VEGF-A, VEGF-B, PlGF and svVEGF. This 180 kD protein is expressed not only in EC, but also in pericytes, placental trophoblasts, vascular smooth muscle, osteoblasts, macrophage/macrophages, renal mesangial cells, certain hematopoietic stem cells and tumour cells. Alternative splicing of the VEGFR-1 gene generates a shorter, soluble variant (soluble flt-1 or sVEGFR-1), which can sequester VEGF and thereby inhibit its action. Expression of the VEGFR-1 gene is induced by hypoxia and activation of the HIF-1 pathway. VEGFR-1 ligands produce a much lower response than that induced by binding to VEGFR-2, particularly in terms of EC proliferation and survival. On the other hand, VEGFR-1 plays a role in the migration of EC and monocytes/macrophages, in hematopoietic stem cell recruitment and in pathological angiogenesis.

2.9. VEGFR-2

VEGFR-2 is a 200–230 kD glycoprotein which binds VEGF-A with lower affinity than VEGFR-1, and also binds VEGF-C and VEGF-D, VEGF-E and svVEGF. It has been shown that VEGFR-2 is the main effector of VEGF-mediated stimulation of EC, particularly in terms of vasodilatation, proliferation and migration. VEGFR-2 is expressed in EC and their circulating precursors, as well as in vascular smooth muscle cells, pancreatic duct cells, some retinal and hematopoietic stem cells and tumour cells. As with VEGFR-1, a truncated form of VEGFR-2 may exert a regulatory effect on VEGF-induced angiogenesis.

2.10. VEGFR-3

VEGFR-3 is a 170–190 kD glycoprotein which binds VEGF-C and VEGF-D. During development it is expressed in all EC subtypes whereas in adults its expression is restricted to lymphatic EC and fenestrated capillaries. It is essential for development of the embryonic vasculature and for lymphangiogenesis. A mutation in this receptor has been implicated in human hereditary lymphedema.

3. Neuropilins

The neuropilins NP-1 (Nrp-1) and NP-2 (Nrp-2) are receptors, which lack an intracellular tyrosine kinase domain. They bind class III semaphorins, which have repulsive effects during axon growth. In order for them to intervene during angiogenesis, they must interact with receptors containing a tyrosine kinase domain, and hence in this respect they are considered as coreceptors. For instance, NP-1 can form a complex with VEGFR-1 and VEGFR-2, which amplifies the binding of VEGF165. NP can be expressed by tumour cells, which explains why VEGF can bind to such cells in the absence of a VEGFR receptor.

4. Regulation of VEGF gene expression

VEGFR gene expression is controlled by a variety of mechanisms, usually involving the protein kinase C, protein kinase A or cAMP pathways [18,34,35,44,99]. Other cAMP-independent mechanisms also play an important role by affect-
ing either VEGF mRNA transcription or mRNA stability [34, 47, 52, 74, 77]. The different factors involved in this regulation include cytokines [20, 25, 77, 92], prostaglandins [52], hormones such as progesterone [57], estrogens [21, 44], gonadotrophins [72], TSH [68] and angiotensin II [120] but also factors as diverse as glucose, protein kinase C modulators, adenylate cyclase activators, nitric oxide, calcium influx, the degree of cellular differentiation, the electron transport chain, depolarizing agents and the level of expression of certain oncogenes. Local or systemic hypoxia is an important mediator of VEGF expression [73, 85, 98], since VEGF acts as an alarm signal for cells “calling for help” in conditions of hypoxic stress. The fact that this is both a nonspecific and general phenomenon explains the ubiquity of VEGF expression. VEGF is induced by HIF-1 (hypoxia-inducible factor 1), a heterodimeric transcription factor, which binds to the VEGF promoter and also enhances VEGF mRNA stability. bFGF upregulates VEGF synthesis in rabbit smooth muscle fibers in synergy with hypoxic stress [108]. Cell differentiation is a major regulatory mediator of VEGF gene expression [18].

Although VEGF stimulates vessel growth and promotes the formation of neovascularization in tumour tissue, it is not an oncogene in and of itself [33]. Nevertheless, at least two oncogenes, src and ras, as well as the tumor suppressor gene p53 participate in regulating its expression. An oncogenic mutation or amplification of ras can stimulate VEGF expression [51, 96] or potentiate hypoxia-induced VEGF expression [80]. The proto-oncogenes cSsrc and Raf-1 appear to play a role in hypoxic induction of VEGF [87], an action, which is inhibited by the p53 tumor suppressor [86]. P53 mutants amplify the angiogenic response, conferring greater resistance to hypoxia, and prevent hypoxic apoptosis induced by non-mutant forms of p53 [63]. Another tumor suppressor, the VHL gene (von Hippel-Lindau), is involved in regulating VEGF expression [103]. Renal tumour cells, which do not express the VHL gene or express an inactive mutant form show increased VEGF expression [103]. It is known that VHL downregulates hypoxia-inducible genes, in particular VEGF. In the presence of inactivating mutations in VHL, the mRNA of these genes can be produced not only during hypoxia but also in normoxic conditions [59].

5. Physiological role of VEGF

5.1. VEGF during embryogenesis

VEGF plays a pivotal role during embryogenesis, as demonstrated by studies in knock-out (KO) mice. The VEGF isoforms in mice contain one less amino acid than their human counterparts. Homozygous VEGF-A<sup>-/-</sup> knockout mice die at E8-E10.5 (day 8 to 10.5 of embryonic life), and heterozygous VEGF-A<sup>+/</sup> mice die at E11-E12, illustrating the critical role of VEGF-A during embryonic development. These mice exhibit profoundly aberrant vascular development and a significant reduction in the erythrocyte cell lineage in the yolk sac. In knock-out mice homozygous for specific VEGF-A isoforms, the effects differ according to the remaining isoform. For example, half of VEGF-A<sup>120/120</sup> mice expressing only the VEGF-A<sub>120</sub> isoform die shortly after birth from multisisceral haemorrhage, while the other 50% die by day 14 of heart failure secondary to ischemic cardiomyopathy. These mice also exhibit less capillary branching as well as skeletal and retinal vascular abnormalities. VEGF-A<sup>188/188</sup> mice suffer from dwarfism, bone and joint ailments as well as abnormal retinal arterial development, whereas VEGF-A<sub>164-164</sub> mice are healthy. The receptors for VEGF-A comprise VEGFR-1, VEGFR-2, VEGFR-1 and VEGFR-2 heterodimers, NRP-1 (VEGF<sub>165</sub>) and NRP-2 (VEGF<sub>145</sub> and VEGF<sub>165</sub>).

Homozygous loss of the VEGFR-1 or VEGFR-2 gene is embryonic lethal between day E8.5 and E9.5, again suggesting a key role for these receptors in vasulogenesis. However, while VEGFR-2<sup>-/-</sup> mice show signs of defective hematopoiesis and die as a result of an underdeveloped vasculature secondary to defective EC growth, VEGFR-1<sup>-/-</sup> mice die from disordered overgrowth of EC, disorganized circulatory development and heart failure. These data suggest that VEGFR-2 is a positive effector of angiogenesis during embryogenesis whereas VEGFR-1 would exert a negative control on vessel growth so that it proceeds in an orderly manner.

Disruption of NRPs, besides causing the neurological lesions linked to their primary function, also results in abnormal embryonic vasulogenesis. Nrp1<sup>-/-</sup> mice are embryonic lethal between day E12 and E13, while the perinatal mortality rate is approximately 40% for Nrp2<sup>-/-</sup> mice and the homozygous double deletion Nrp1<sup>-/-</sup>, Nrp2<sup>-/-</sup> causes intrauterine death at day E11 to E12.

5.2. Physiological roles of VEGF during the postnatal period

VEGF is involved in the correct functioning of two important physiological processes: wound healing [16] and the menstrual cycle.

5.2.1. VEGF and wound healing

As a consequence of injury, platelets, activated by the subendothelium, release cytokines, which initiate the process of clot formation and wound healing. Thrombocytes also release VEGF, which recruits circulating neutrophils and monocytes to the site. These monocytes as well as local keratinocytes and EC release VEGF, which stimulates local angiogenesis, vasculogenesis and microcirculatory permeability and may stimulate the formation of granulation tissue. VEGF is also a stimulator of pericytes, which line the newly formed vessels. It also promotes consolidation of bone fractures.

5.2.2. VEGF and the menstrual cycle

VEGF plays a role in the vascular processes involved in the normal menstrual cycle. VEGF expression peaks during the early luteal phase, when new vessel growth must be most intensive for proper development of the corpus luteum. VEGF expression then decreases during the progestative phase and stops just before menstruation.
6. Role of VEGF in pathological processes

6.1. VEGF and tumour pathology

There is ample experimental evidence pointing to a correlation between tumour growth, metastasis [26] and the growth of blood vessels [9,90,111]. Most human cancers, which have been studied express VEGF mRNA and/or show increased production of VEGF [14,30,31].

6.2. VEGF and non-tumoral pathologies

VEGF plays a role in diabetic retinopathy and other retinal disorders with an ischemic component, such as macular degeneration [1,2,79,84,91,93]. Local production of VEGF stimulates retinal neovascularization and retinal vascular permeability, leading to exudate production and the risk of retinal detachment due to traction of the neovessels. These phenomena can be compared to the retinopathy observed in premature infants on respiratory assistance. When normal oxygenation resumes, the retina, which is in relative hypoxia, stimulates local production of VEGF and IGF-1, particularly by astrocytes and Muller cells, resulting in proliferation of retinal vessels. VEGF is also involved in delayed hypersensitivity reactions [15], inflammatory disorders such as rheumatoid arthritis [27,71] or psoriasis [23], endometriosis [82,83,102], ovary hyperstimulation syndrome [81], eclampsia [4], polycystic ovary [61] as well as coronary disease and peripheral arterial disease via the processes of atherosclerosis [89] and intima hyperplasia.

7. VEGF and the thyroid gland

7.1. VEGF expression in normal thyroid

VEGF is found in almost all body tissues, although the levels of expression differ according to the tissue and the state of tissue maturation. We found that VEGF was expressed in all normal thyroid tissue samples studied [69]. This expression appeared to be specific for thyrocytes, since VEGF mRNA and protein immunostaining were only seen in thyroid epithelial cells, with no labelling observed in fibroblasts. These data corroborate earlier findings [113]. The endocrine function of the thyroid probably requires a precise regulation of its microvasculature. The proximity of VEGF-synthesizing thyrocytes to the capillary endothelial cells which bind to VEGF via high-affinity receptors on the endothelial surface of their cytoplasmic membrane suggests a paracrine regulation of local angiogenesis between these two cell types.

Cultured thyrocytes are capable of synthesizing and secreting VEGF-A in vitro. VEGF-A mRNA synthesis is regulated by factors controlling cAMP and protein kinase signalling pathways such as TSH or the thyroid stimulating IgG immunoglobulins underlying the autoimmunity of Graves’ disease, dibutyryl cAMP, or the serum of Graves’ disease patients [99]. The regulatory role of TSH on thyroid cells has been confirmed in vitro on cancer cell lines [106]. Along the same lines, we recently showed that rhTSH could stimulate thyroid angiogenesis in vivo, probably as a result of increased VEGF expression in thyrocytes [68]. On the other hand, TSH and VEGF appear to exert an antagonistic regulatory control of iodine uptake or tritiated thymidine incorporation by thyroid follicular cells.

Lastly, while so far there is no evidence that VEGF receptors are expressed in human thyroid cells, Wang et al. detected VEGFR-1 mRNA in rat thyroid cells using RT-PCR [115].

7.2. VEGF expression in thyroid pathologies

7.2.1. Thyroid cancer

In 1971, Judas Folkman postulated that a tumour or metastasis could only develop if new vessels grew out from healthy tissues to nourish the cancerous cells [36]. When it reaches a volume of more than one cubic millimeter, the tumour elaborates its own nutritional system and stimulates the growth of new vessels towards the tumour tissue [36,39,41]. This step is an essential condition for tumour development [46]. Observations of tumour architecture confirm that malignant cells are never too far away from a capillary structure [110], at a distance, which in principle never exceeds the limit of diffusion of macromolecules. The acquisition of a large vascular infrastructure not only serves to nourish the tumour, but also promotes tumour growth [13,37,46] by conveying growth factors to the very site of tumour development and favouring metastatic dissemination [10,107,119]. In papillary thyroid carcinoma, microvascular density can double or triple [48] with respect to normal thyroid tissue [3]. This might reflect the metastatic potential of a tumour and serve as a prognostic marker for different cancers including breast [53,118], prostate [43,117], lung [123], head and neck [45] or melanoma [50,107]. This prognostic role has been partly confirmed for differentiated thyroid carcinomas [24,60] and medullary thyroid carcinoma [42], although the data are discordant in the smaller studies [3,48]. These conflicting data may arise from methodological differences in the evaluation of microvascular density [75]. At least two studies have reported surprising results in so far as microvascular density was smaller in the least differentiated and therefore presumably most aggressive cancers [3,54].

High levels of VEGF production in malignant thyroid cells in differentiated and medullary thyroid carcinomas are therefore expected and has been confirmed in several studies [17,62,69,105,112]. However VEGF expression in the cancerous thyroid is not uniform. Intense immunostaining is seen within the transformed cells and in the thyroid vesicles immediately surrounding the tumour, but then it gradually tapers off without a clean transition line [69]. This very gradual cellular gradient is seen in both immunohistochemical studies with the protein and by in situ hybridization using VEGF mRNA. This suggests a paracrine stimulation of VEGF either by diffusible substances secreted by the neoplastic cells or their environment, or the response to a hypoxic gradient. Indeed, the tumour is the site of intense proliferative activity, which generates relative or
complete hypoxia in the most active area as compared to more distant areas. The necrotic foci frequently seen within tumours are a manifestation of this hypoxia. The very high levels of VEGF expression would thus reflect the response to this hypoxic stress. As the distance from the most active areas increases, hypoxia would diminish and cells would express less and less VEGF. Normal, non-hypoxic tissues farthest from the tumour show normal levels of VEGF expression. Here again, the absence of VEGF mRNA within EC argues against an autocrine regulation of angiogenesis by EC in thyroid carcinoma. Moreover, regardless of the tumour type, the VEGF receptors Flt-1 and Flk-1/KDR are more highly expressed in EC of tumour vessels than in EC of nearby normal tissue [94,116]. Viglietto et al. showed that a high tumorigenic potential was correlated with high VEGF expression in human thyroid tumours [113]. These authors found higher VEGF expression in all anaplastic thyroid carcinomas than in differentiated papillary or follicular carcinomas. Other studies have confirmed a significantly higher level of VEGF expression in papillary and follicular thyroid carcinoma than in normal thyroid tissue [69,105], as well as in medullary thyroid carcinoma [69]. On the other hand, VEGF expression levels appear similar in both the primary tumour and metastatic foci, whether lymphatic or pulmonary [105].

While increased VEGF expression in thyroid cells is not synonymous with cancer, high levels of VEGF expression in thyroid carcinoma may, on the other hand, be an indicator of tumour aggressiveness and therefore serve as a prognostic marker. For instance, large or metastasized tumours have higher levels of VEGF expression [17,64], including in children [29]. A higher VEGF immunostaining score was also found in thyroid carcinomas that had metastasized [70]. In papillary thyroid carcinoma, the metastatic risk associated with high VEGF expression encompasses both locoregional lymphatic spread as well as metastasis via blood vessels [76]. The VEGF isoforms VEGF-C and VEGF-D, involved in lymphangiogenesis, have been incriminated in metastasis to the cervical lymph nodes in papillary cancer, which has long been known to favour the lymphatic system [109,124,128]. It is also noteworthy that VEGF-C expression is higher in papillary carcinoma than in follicular carcinomas, which tends to metastasize via the blood [28]. Yasouka et al. established not only that VEGF-D is a risk factor for lymph nodes metastasis, but that this risk is also correlated with lymphatic microvascular density [124].

VEGF plasma concentrations can be determined by immunoenzymatic methods. Several studies have reported higher serum VEGF levels in patients with differentiated thyroid carcinoma as compared to patients with a normal thyroid or a simple goitre [78].

Nevertheless, while VEGF can be considered an independent prognostic factor, it cannot on the other hand be considered a marker of thyroid cancer when found in abundance in thyroid tissue. This is because VEGF expression in Graves’ disease, Hashimoto’s chronic lymphocytic thyroiditis or some toxic adenomas can be just as high as in thyroid carcinoma [69]. Consequently, it is impossible to distinguish between benign and malignant disease simply on the basis of increased VEGF expression. The reverse is also true, since some carcinomas were found to have low VEGF immunostaining scores, even lower than the scores from normal thyroid. These patients are still in remission at the present time, in some cases more than 20 years after diagnosis. This might lead to the speculation that a low VEGF immunostaining score might reflect low aggressiveness, even for malignant lesions. However, it would be unwise to establish such a rule on the basis of a just two cases.

7.2.2. VEGF as therapeutic target in thyroid carcinoma

One line of evidence that angiogenesis plays an important role in tumour expansion is that it is reduced after neutralization of VEGF by specific monoclonal antibodies [65,66,116]. Injection of these antibodies leads to a rarefaction of vessel branching and a lower vascular density [66,116]. These data have been confirmed by newer in vivo microscopy techniques [11,12].

Many other angiogenesis inhibitors have since been developed for therapeutic purposes. In most cases these are anti-VEGF monoclonal antibodies or tyrosine kinase inhibitors acting on VEGF receptors (Table 1). Data from animal studies are encouraging. For example, the anti-VEGF monoclonal antibody mAbA4.61 reduced tumour volume and tumour vascular density in nude mice implanted with FTC-133 cells [104]. Six weeks of treatment with an anti-VEGF monoclonal antibody led to a 60% decrease in tumour volume after subcutaneous implantation of ARO-81 cells in nude mice [6]. The absence of any in vitro activity on the cell line indicates that this is not a direct cytotoxic effect but rather occurs through inhibition of a factor secreted in vivo, in this case VEGF. The same authors confirmed their findings in the case of intraperitoneal administration [5].

Along other lines, the VEGFR and EGFR tyrosine kinase inhibitor AEE788 significantly decreased tumour growth in nude mice implanted with WRO follicular thyroid carcinoma cells [127]. Similarly, four weeks of treatment with the VEGF receptor inhibitor PTK 787/ZK222584 given orally at a dose of 75 mg/kg led to a 41% reduction in tumour volume and microvascular density in nude mice implanted with MC-1 follicular carcinoma cells [100]. Manumycin, a farnesyl transferase inhibitor, coadministered with paclitaxel inhibited the growth of an anaplastic carcinoma in nude mice [122].

Other avenues of research have also proved promising. Transfection of ARO cells with an antisense transcript inhibited tumour development and vascularization [7], whereas the soluble form of the flt-1 receptor (sFlt-1), by trapping bioavailable VEGF, reduced mean vascular density and decreased tumour volume by 70% in nude mice implanted with FTC-133 cells [125].

Some molecules are now in clinical phase II trials in France. One of them is all the more interesting in that it induced “thyroid disappearance” or hypothyroidism in patients being treated for gastrointestinal or renal cancers [22,97].
7.2.3. Benign thyroid disorders

The angiogenic process is activated in at least two thyroid pathologies: goitre formation and thyroiditis, in particular Graves’ disease.

7.2.3.1. Goitre formation. The involvement of VEGF in thyroid tumour pathology is not restricted to malignant tumours. Different authors have quantified VEGF expression in benign proliferative thyroid disorders. In a study published 30 years ago, Wollman et al. showed that thyroid enlargement during experimentally induced goitre was correlated with a parallel development of the vasculature. The mitotic activity of EC increased to levels even above those of the thyrocytes [121]. In 1987, Goodman and Rone showed that thyroid cells was increased to levels even above those of the thyrocytes [114]. Increased VEGF expression has also been described in Graves’ disease (261 ± 157 ng/l), Hashimoto chronic lymphocytic thyroiditis (300 ± 93 ng/l) and subacute thyroiditis (506 ± 20 ng/l) as compared to healthy controls (130 ± 85 ng/l) [58]. Patients with Hashimoto’s chronic lymphocytic thyroiditis and Graves’ disease [69]. Thyroiditis develops in an autoimmune context associated with the production of blocking antibodies. Despite the frequent hypothyroid context, we have found fairly high levels of VEGF mRNA, which in any case were higher than in normal thyroid tissue samples. This apparent paradox is resolved if one recalls that angiogenesis in general and VEGF in particular are involved in inflammatory reactions [101,126]. Inflammatory cytokines including those involved in thyroiditis are mediators of VEGF expression. The interleukins (IL) are a case in point. IL-6 induces VEGF expression in different cell lines [19] and IL-1β stimulates VEGF mRNA expression in rat aortic smooth muscle cells [77]. IL-2 and PGE2 both stimulate VEGF synthesis in cultured synovial fibroblasts, suggesting an identical mechanism of stimulation in joint inflammation [8].

In addition to higher local expression of angiogenic factors, serum VEGF is also increased. A Japanese study compared VEGF serum concentrations in 49 patients with Graves’ disease, 23 with subacute thyroiditis, 24 with Hashimoto’s chronic lymphocytic thyroiditis and 37 healthy controls. The authors found significantly higher mean VEGF serum levels in Graves’ disease (261 ± 157 ng/l), Hashimoto’s thyroiditis (300 ± 93 ng/l) and subacute thyroiditis (506 ± 20 ng/l) as compared to healthy controls (130 ± 85 ng/l) [58]. Hashimoto patients also had a significantly higher intrathyroidal vascular area, in contrast to those with subacute thyroiditis. However, the latter group had higher serum VEGF levels than any other group (P < 0.0001) [58]. Patients with Hashimoto’s thyroiditis were given levothyroxin and those with subacute thyroiditis received prednisolone, which led to a significant reduction of VEGF levels in both groups. Serum concentrations dropped to 163 ± 135 ng/L in the first group and to 172 ± 114 ng/l in the patients with subacute thyroiditis [58]. The authors concluded

### Table 1
Inhibitors of the VEGF and its receptors in on-going studies

<table>
<thead>
<tr>
<th>Name of inhibitor</th>
<th>Therapeutic class</th>
<th>Target</th>
<th>Pharmaceutical company</th>
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<td>Bevacizumab</td>
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<td>VEGF-A</td>
<td>Genentech</td>
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<td>Monoclonal Ab</td>
<td>VEGFR-2</td>
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<td>Monoclonal Ab</td>
<td>VEGF-A</td>
<td>Peregrine</td>
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<td>Novartis</td>
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<td>Novartis</td>
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<td>Tyrosine kinase inhibitor</td>
<td>VEGFR-1, -2, PDGF</td>
<td>Pfizer</td>
</tr>
<tr>
<td>Axitinib</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CEP-7055</td>
<td>Tyrosine kinase inhibitor</td>
<td>VEGFR-1, -2, -3</td>
<td>Cephalon</td>
</tr>
<tr>
<td>CP-547, 632</td>
<td>Tyrosine kinase inhibitor</td>
<td>VEGFR-1, -2</td>
<td>Pfizer</td>
</tr>
<tr>
<td>VEGF-trap</td>
<td>Soluble receptor</td>
<td>VEGF-A, PI GF</td>
<td>Aventis/Regeneron</td>
</tr>
<tr>
<td>GW76024 Pazopanib</td>
<td>Tyrosine kinase inhibitor</td>
<td>VEGFR-1, -2, -3 PDGFR, c-kit</td>
<td>Glaxo Smith Kline</td>
</tr>
<tr>
<td>Bay 93-4006, Sorafenib</td>
<td>Tyrosine kinase inhibitor</td>
<td>VEGFR-1, -2, -3 PDGFR, Raf1, MAPK, erk, c-kit</td>
<td>Bayer/Onyx</td>
</tr>
<tr>
<td>AMG-706 Motesanib diphosphate</td>
<td>Tyrosine kinase inhibitor</td>
<td>VEGFR-1, -2, -3 PDGFR, RET, c-kit</td>
<td>Amgen</td>
</tr>
</tbody>
</table>

- Quervain’s subacute thyroiditis,
- Hashimoto’s chronic thyroiditis
- Graves’ disease [69].
that VEGF produced by thyroid epithelial cells plays an important role in intrathyroidal angiogenesis in patients with Graves’ disease or Hashimoto’s thyroiditis with hypothyroidism and a goitre. In contrast, the high levels of VEGF found in subacute thyroiditis would be related to systemic inflammation and not to inflammation of the thyroid [58].

7.2.3.3. Graves’ disease. Angiogenesis is highly stimulated in Graves’ disease, which produces a vascular goitre giving a bruit on auscultation.

VEGF is an important effector of the angiogenic process. Increased expression of VEGF, PIGF and the receptors VEGFR-1 and VEGFR-2 has been demonstrated in this disease [69,88,114]. The thyroid stimulating antibodies underlying Graves’ disease stimulate VEGF expression [99]. A strong correlation has been found between intrathyroidal vascular area determined by Doppler velocimetry and VEGF serum concentrations in untreated Graves’ disease patients with a goitre larger than 40 cm³ (R = 0.877; P = 0.0042) [58]. A similar correlation has been reported between hypervascularity and expression of VEGF and its type I receptor (flt-1) [88]. Treatment with synthetic antithyroid drugs lowers both thyroid vascularization and serum VEGF [58]. One study found no difference in VEGF-C expression between normal and Graves’ disease thyroid tissue, although the sample size in this study was very small [56].

The control of VEGF expression in benign thyroid diseases probably involves the physiological endocrine regulation of the thyroid gland. Of course this control is not necessarily direct and we would even speculate that it is probably indirect. TSH (in hypothyroidism, for example secondary to thyroiditis) and thyroid stimulating antibodies (TRAK, Tsab, etc.) in Graves’ disease are excellent candidates for stimulating VEGF production by thyrocytes [68,99]. The stimulatory role of these two types of peptide on thyroid growth and function is well established. In our opinion, their potential role in paracrine stimulation of thyrocytes cannot be ruled out.

In summary, increased VEGF expression is observed in both benign and malignant disease processes of the thyroid gland. While in the latter case high VEGF levels may be a predictor of a poor outcome, high VEGF expression alone does not constitute evidence for malignancy. VEGF expression is probably under dual paracrine and endocrine control, particularly through the action of TSH.

French version

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