REVIEW

Vascular Endothelial Growth Factors and Thyroid Disorders

KANJI SATO

Department of Medicine, Institute of Clinical Endocrinology, Tokyo Women's Medical University, Tokyo 162–8666, Japan

Key Words: Vascular endothelial growth factor (VEGF), Cyst fluid, Graves' disease, Thyroid carcinoma, Iodide

Introduction

AS a clinical endocrinologist chiefly seeing patients with Graves' disease and thyroid nodules, I have asked myself several questions 1) why are the thyroid glands of untreated thyrotoxic Graves' patients so hypervascular that bruits are audible on the enlarged thyroid gland, 2) why do certain thyroid cyst fluids disappear or recur after aspiration, 3) why do papillary thyroid carcinomas preferentially metastasize lymphogenously, whereas thyroid follicular carcinomas do so hematogenously, 4) why does large dose iodide, when administered to thyrotoxic patients with Graves' disease prior to subtotal thyroidectomy, decrease the thyroid blood flow. Using thyroid follicles in suspension culture, in which thyrocytes maintain thyroid function very efficiently, we may have answers to some of these questions. In this review, we reinforce the view that vascular endothelial growth factor (VEGF) family [1] is at least partly involved in these phenomena. The elucidation of the regulatory mechanism of thyroid blood flow is likely contribute to the development of new strategies for inhibiting the growth of hypervascular thyroid carcinoma in the near future.

1) Why arterial bruits are audible on the hyperplastic thyroid gland of Graves' patients

When untreated thyrotoxic Graves' patients are

seen on the first visit, the thyroid glands are diffusely enlarged, and arterial bruits are usually audible on the thyroid gland. The increased blood flow can be visually demonstrated as thyroid inferno using color Doppler ultrasonography [3, 4]. This pathognomonic sign of Graves' disease is, however, not due to thyrotoxicosis *per se*, because bruits are also audible in Graves' patients with severe hypothyroidism who had been overtreated with antithyroid agents. These clinical findings suggest that thyroid blood flow is not regulated by serum levels of thyroid hormone, but by certain stimulators on thyrocytes.

In the late 1970s, Dr. S. H. Wollman, at the National Cancer Institute, the National Institutes of Health (NIH), Bethesda, MD, conducted a series of pioneering works using hyperplastic, hypervascular thyroid gland of rats which had been fed antithyroid agents [5–7]. When rats were fed thiouracil for a prolonged period, the thyroid gland became hypervascular on day 3, and gradually enlarged. Electron-microscopic examination revealed that when the thyroid gland starts to enlarge, the first cells that start to proliferate are not the thyrocytes but the endothelial cells surrounding the thyroid follicles (Fig. 1). Capillaries were clearly enlarged by day 3 and they enlarged progressively thereafter. By day 3, the cells of many neighboring capillaries came into close apposition and from this time on, there was evidence of fusion of capillary walls in the form of partial septa in the capillary lumens. Fusion continued until day 20, and the vascular lumen increased to a greater size. Interestingly, vascular enlargement was restricted to the thyroid blood vessels. There was no obvious enlargement of the blood capillaries of the parathyroid despite its close proximity to the thyroid. Wollman therefore postulated that stimulated thyrocytes produce unidentified factor or factors which

Correspondence to: Kanji SATO, M.D., Department of Medicine, Institute of Clinical Endocrinology, Tokyo Women's Medical University, Kawada-cho 8–1, Shinjuku-ku, Tokyo 162–8666, Japan
e-mail: sttokan@attglobal.net

Fig. 1. Blood capillary enlargement during the development of thyroid hyperplasia in thiouracil-fed rats. Rats were fed chow containing thiouracil for 28 days. On days 0, 3, 5, and 21 days, rats were sacrificed and thyroid glands were perfused and embedded into eposin, and examined by electron-microscope. On day 3, when serum levels of TSH increased, endothelial cells started to proliferate (the arrow). On day 5, endothelial cells fused together (the arrow), and on day 21, the blood capillaries surrounding the thyroid follicles were extensively enlarged. Reproduced from Ref. 5, with permission of the publisher.

stimulate endothelial cell proliferation. As a post-doctoral research associate in Dr. Jacob Robbins' laboratory, Clinical Endocrinology Branch, I was greatly impressed by his fascinating slides presented at the Journal Club, NIH, in 1979.

In the following 20 years, a number of angiogenesis factors have been identified, and their receptors also have been cloned [8]. Among the many angiogenesis factors, vascular endothelial growth factor (VEGF) is regarded as the most important because it has a signal peptide, and exclusively stimulates the proliferation of endothelial cells most potently. On the assumption that VEGF is involved in the hyper-vascularity of Graves' disease, we cultured thyroid follicles, obtained at subtotal thyroidectomy from patients with Graves' disease, in serum-free (or 0.2% fetal bovine serum-containing) medium in agarose-coated culture dishes [9]. In agarose-coated dishes, thyroid follicles cannot attach to the bottom of culture dishes and can be cultured in suspension for a

couple of weeks maintaining thyroid function such as ^{125}I incorporation and release of *de novo* synthesized thyroid hormones (^{125}I -T3 + ^{125}I -T4) into the culture medium (Fig. 2) [10–12]. When thyroid follicles were cultured in the presence of TSH for a couple of days, steady state mRNA levels of VEGF were increased several fold in thyroid follicles cultured in low concentrations of fetal bovine serum (0.5%) (Fig. 3) [13]. The same phenomenon was also observed in thyroid follicles cultured with Graves' IgG. Since VEGF mRNA expression is stimulated by dibutyryl cyclic AMP, forskolin, calcium ionophore, phorbol esters, it was concluded that once TSH receptors are stimulated by TSH or TSH receptor antibodies, the adenylate cyclase and protein kinase C pathways are stimulated, followed by increased expression levels of VEGF. Since VEGF has a signal peptide, it is secreted from thyroid follicles, and activates VEGF receptors on endothelial cells in a paracrine fashion.

Fig. 2. Culture methods for human thyroid follicles. Thyroid follicles in suspension culture (left) and monolayer culture (right). Thyroid follicles (TF) were prepared from thyroid tissues obtained from Graves' patients at subtotal thyroidectomy. TF were cultured in tissue culture dishes which had been coated with (left) or without agarose (right). TF cultured in the coated dishes could not attach to the bottom and were cultured in suspension, maintaining thyroid function for a couple of weeks. When ¹²⁵I was added, TF incorporated ¹²⁵I and secreted *de novo* synthesized thyroid hormones (¹²⁵I-T₃ + ¹²⁵I-T₄) into the culture medium, in response to Graves' IgG and bTSH even at 0.1 μ U/ml, thus being the most sensitive bioassay for Graves' IgG (57). In a few experiments, TF were cultured in dishes which had not been coated with agarose so that the culture medium could be changed completely and frequently (48). Under such a culture condition, TF attached on the bottom of culture dishes, gradually forming a monolayer of thyrocytes (right). Although the sensitivity to TSH was greatly impaired, these thyrocytes could synthesize thyroid hormones for a couple of weeks.

This story was confirmed by *in vivo* experiments. Following the Wollman experiments exactly [5], rats were fed thiouracil-containing chow for 28 days. Serum levels of T₃ and T₄ decreased by day 3, accompanied with rapidly increasing serum levels of TSH. VEGF mRNA expression level peaked on day 3 in the hypervascular thyroid gland, accompanied with increased expression levels of VEGF receptors, namely Flt-1 (VEGFR-1) and KDR (VEGFR-2) (Fig. 4) [13]. For unknown reasons, the expression levels of VEGF and VEGFR gradually decreased thereafter, although the hypervascularity of the thyroid gland persisted. In accordance with these experimental data, VEGF and its receptor (VEGFR-1) mRNA were shown to have increased in hyperplastic thyroid gland in Graves' disease [14, 15].

Therefore, we speculate that when TSH receptor is stimulated by TSH or Graves' IgG, VEGF mRNA

expression is increased via the adenylate kinase and protein kinase C pathways. VEGF is then secreted from thyroid follicles and stimulates VEGF receptors on the endothelial cells in a paracrine fashion. Endothelial cells, once their VEGF receptors are stimulated, start to proliferate and fuse to each other, so that vascular lumen increased (Fig. 5). According to Poiseuille's law, when a lumen increases 2-fold, flow volume increases tremendously. In addition, cardiac output in Graves' patients is increased so that thyroid blood flow, which is the most abundant in the whole body (4–6 ml/min/g) [16], increases to a greater extent, leading to arterial bruits in Graves' patients. Our findings were confirmed by Viglietto *et al.* [14], who found that, in addition to VEGF, production of a VEGF-related angiogenesis factor, placenta growth factor (PlGF), is also enhanced by TSH, and demonstrated that both angiogenesis fac-

Fig. 3. VEGF mRNA expression in thyroid follicles cultured with or without TSH. Human thyroid follicles were cultured in F-12/RPMI-1640 medium supplemented with bovine TSH (bTSH, 10 μ U/ml) and various concentrations of fetal bovine serum (FBS). After 3 days of culture, total RNAs were electrophoresed and hybridized with 32 P-labelled VEGF cDNA, and subsequently with 32 P-labelled GAPDH cDNA. Note that VEGF mRNA expression is remarkably induced by TSH in thyroid follicles cultured with low concentrations of FBS (0.5%). Reproduced from Ref. 13, with permission of the publisher.

tors are upregulated by TSH at the transcriptional level using run-on transcription assay on nuclei isolated from cultured rat thyrocytes (PC Cl 3 cells). TSH-mediated VEGF production was also observed in a thyroid carcinoma cell line that maintains functional TSH receptors [17].

It is well-known among thyroidologists practicing echography that thyroid blood flow is increased in some patients with Hashimoto's thyroiditis, whose serum levels of TSH increase up to 100 μ U/ml. The increased blood flow decreases after supplementation of L-thyroxine, indicating that TSH is partly involved in hypervascularity of Hashimoto's thyroid gland [18]. In view of the fact that several pro-angiogenic cytokines (IL-1 α , TNF- α) induce VEGF mRNA expression [19], it is also possible that VEGF mRNA is induced by these proinflammatory cytokines, which are known to be produced in autoimmune thyroid disease [20]. Indeed, increased production of VEGF-A was demonstrated in Hashimoto's thyroiditis [21].

More recently, Ramsden *et al.* [22] reported that Tie-2 (a tyrosine kinase receptor for the angiogenesis factor, angiopoietin-1), which is usually expressed only in endothelial cells in adult tissues, is also ex-

pressed on thyroid follicular cells. Furthermore, Tie-2 expression is increased in goiter, and is up-regulated by TSH, suggesting that angiopoietin-1 and Tie-2 system are involved not only in goitrogenesis but also in angiogenesis in the thyroid gland [23].

2) Why certain thyroid cyst fluids disappear or recur after aspiration

A number of patients visit our thyroid clinic with chief complaints of thyroid nodules, such as adenomatous goiter or follicular adenoma with cystic degeneration.

When a cyst fluid is aspirated, the nodules may decrease in size or completely diminish in a few patients, but it recurs again in many patients in a few weeks. Since human thyrocytes produce VEGF, which is also purified as vascular permeability factor (VPF), VEGF/VPF concentration in cyst fluid was determined. Although there was no significant difference of VEGF/VPF concentration in the cyst fluid obtained from thyroid adenoma or from adenomatous goiter, it was significantly increased in the cyst fluid obtained from patients who required repeated aspiration (84.8 ± 58.3 ng/mL, mean $\pm$ SD, $n=18$) than that in the cysts that regressed or disappeared after a single aspiration (4.3 ± 4.4 ng/mL, $n=12$, $P<0.001$) (Fig. 6) [24]. The immunoreactive VEGF/VPF in cyst fluid was eluted mainly at 45 kDa on gel-filtration, and stimulated endothelial cell proliferation, which was partially blocked by anti-VEGF/VPF antibody. Therefore, VEGF/VPF is at least partly involved in the accumulation of cyst fluid in thyroid nodules, although other permeability factors, such as VEGF-C, may also be involved. We recommend therefore that when cyst fluid reaccumulates rapidly after its aspiration and the VEGF/VPF concentration is high in the cyst fluid, it is better for the patients to receive an interventional treatment, such as percutaneous ethanol injection therapy (PEIT).

3) Why thyroid papillary carcinomas metastasize lymphogenously

In general, neoplastic cells which express VEGF family are clinically aggressive; they grow rapidly and easily metastasize to distant organs. As expected,

Fig. 4. Thyroid function and mRNA expression levels of VEGF and VEGFR in the thyroid gland of thiouracil-fed rats. Rats were fed thiouracil-mixed chops for 28 days. On days 0, 3, 7, 14, 21 and 28 days, they were sacrificed and their serum levels of T₃, T₄ and TSH were determined by radioimmunoassay (lower panel). Total RNA was prepared from thyroid glands, and mRNA expression levels of VEGF and its receptors (Flt-1, KDR) were determined by Northern blot hybridization. Data were presented as the ratio compared with that of β -actin mRNA (upper panel) (modified from Ref. 13).

Fig. 5. Pathogenesis of increased blood flow in the thyroid gland in patients with Graves' disease.

Fig. 6. VEGF/VPF levels in cyst fluids in the thyroid nodules.

this is also the case in thyroid carcinomas [25–30]. Indeed, VEGF is most abundantly produced by highly malignant anaplastic carcinomas. Papillary and follicular carcinomas express intermediate levels of VEGF mRNA [25]. Increased expression of VEGF, which correlates with the size of papillary carcinoma in children, is a hallmark of a trend to

produce metastasis [29], and associated with increased risk of recurrence and decreased disease-free survival in papillary thyroid cancer [30].

It is well known that papillary carcinomas metastasize lymphogenously whereas thyroid follicular carcinoma hematogenously [31]. For cancer cells to metastasize, the cells need to enter the circulation, attach to the endothelia of blood vessels or lymphatics, and invade into distant organs or regional lymph nodes. Recently, new VEGFs were identified, and named as PlGF, VEGF-B, VEGF-C, and VEGF-D [32, 33]. The original VEGF/VPF was referred to as VEGF-A [1]. Each VEGF has its own VEGF receptor (VEGFR), namely Flt-1 (VEGFR-1), KDR (VEGFR-2), and Flt-4 (VEGFR-3). VEGF-A activates VEGFR-1 and 2 and stimulates endothelial cells to proliferate, leading to angiogenesis (Fig. 7), whereas both PlGF and VEGF-B activate only VEGFR-2 and generate a weak signal to angiogenesis. In contrast, VEGF-C activates VEGFR-2 and VEGFR-3, leading to angiogenesis and lymphangiogenesis, respectively [32, 33].

On the assumption that VEGF-C may be involved in the process of lymphatic metastasis of papillary carcinoma, VEGF mRNA expression levels were studied using Northern blot analysis. VEGF-C gene expression was significantly increased in the papillary more than in the follicular carcinoma [34]. In a few

Fig. 7. Roles of VEGF family and their receptors in thyroid disorders. Once TSH receptors are stimulated by Graves' IgG, production of VEGF is enhanced via the adenylate kinase A and protein kinase C pathways. VEGF stimulates VEGF receptors of the endothelial cells in a paracrine fashion, leading to angiogenesis. In papillary carcinoma, production of VEGF-C is increased. VEGF-C activates VEGFR-3, leading to lymphangiogenesis, promoting lymphatic metastases to the regional lymph nodes.

cases, nonmalignant thyroid tissue from patients with papillary carcinoma also expressed higher levels of VEGF-C than tissue from patients with follicular carcinoma. Expression of the VEGF-C gene was observed by *in situ* hybridization in cells of papillary thyroid carcinoma but not in those of follicular carcinoma (Fig. 8). These findings suggest that increased expression of the VEGF-C gene by both tumor cells and the surrounding tissue may be related to the prevalence of intrathyroidal spread through lymphatics and regional lymph node. Similar findings were reported simultaneously [27, 35]. These findings are clinically very important since VEGF-C not only stimulates lymphangiogenesis but also promotes intralymphatic tumor growth [36]. Furthermore, overexpression of VEGF-C in breast cancer cells or in transgenic mice increased intratumoral lymphangiogenesis and enhanced metastasis to regional lymph nodes [37, 38]. Increased expression of VEGF-C in tumors with lymph node metastasis has been reported in head and neck carcinomas, gastric carcinoma, esophageal carcinoma, pulmonary carcinoma, cervical carcinoma and prostatic carcinoma [39–44]. It is interesting to note that thyroid carcinomas with distant hematogenous metastases express increased mRNA levels of angiogenesis factors (VEGF and angiopoietin-2) and decreased levels of angiogenesis inhibitor (thrombospondin-1) [27].

Since VEGF-C is also capable of stimulating angiogenesis and lymphangiogenesis *in vivo* [45], and since Graves' thyroid gland is abundant in lymph vessels, we investigated VEGF-C mRNA expression in markedly enlarged Graves' thyroid glands. In addition to VEGF-A mRNA expression, VEGF-C

mRNA is highly expressed in proliferating thyrocytes whereas VEGFR-3 is expressed exclusively in the lymphatic and vascular endothelium (Kato Y *et al.*, unpublished observation). These findings indicate that VEGF families are involved in the angiogenesis and lymphangiogenesis in Graves' disease as well as in neoplastic diseases of the thyroid gland. The regulatory mechanism of VEGF-C expression is largely unknown, but several cytokines may certainly be involved for VEGF-C mRNA expression [46].

4) Why high dose iodide decreases thyroid blood flow in Graves' patients

In patients with thyroid storm, a large amount of iodide is routinely administered to inhibit thyroid hormone synthesis and release from thyroid gland. This phenomenon, acute Wolff-Chaikoff effect, can be reproduced in porcine and human thyroid follicles *in vitro* [47, 48] (Fig. 9). This pathogenesis was extensively reviewed by Wolff himself [49]: iodolactones derived from arachidonic acid and iodoaldehydes derived from plasmalogens are regarded as the candidates, but additional factors, such as TGF- β , may be also involved. Production of TGF- β is reportedly enhanced by iodide at high concentrations [50], but there are controversial reports [51]. Furthermore, sodium/iodide symporter (NIS), whose mRNA expression is induced by TSH and decreased by a high dose of iodide [52], is also involved in iodine-induced inhibition of thyroid hormonogenesis and escape phenomenon from the acute Wolff-Chaikoff effect.

A large amount of iodide is also prescribed for

Fig. 8. Expression of VEGF-C mRNA in thyroid carcinoma by *in situ* hybridization. Expression of the VEGF-C gene was observed by *in situ* hybridization in cells of papillary thyroid carcinoma (left panel) but not in those of follicular carcinoma (right panel). Reproduced from Ref. 34, with permission of the publisher.

Fig. 9. Effect of NaI concentrations on T3 production in human thyrocytes in follicle culture. Thyroid follicles were cultured in 24-multiwell dishes in supplemented culture medium containing various concentrations of NaI with or without additional bTSH (100 μ U/ml), for up to 16 days. After 2 days of preculture, the medium was changed every 3 days (except days 9–13) for 14 days. Data are means $\pm$ SD of triplicate cultures. $\blacktriangle$ — $\blacktriangle$, days 3–5; $\square$ — $\square$, days 6–8; $\bullet$ — $\bullet$, days 9–13; $\circ$ — $\circ$, days 14–16. Reproduced from Ref. 48, with permission of the publisher.

patients with Graves' disease 7–14 days before subtotal thyroidectomy to decrease thyroid blood flow [53, 54]. This medication has been routinely performed for the last 50 years; however, the precise mechanism has not been well elucidated.

Recently an increasing number of angiogenesis factor and its inhibitor were reported [2]. It could be postulated that iodide, at high concentrations, either decreases some angiogenesis stimulators or increases angiogenesis inhibitors, or both (Fig. 10). Presumably, the main factor is not VEGF-A, since VEGF-A mRNA expression was only transiently reduced at extremely high iodide concentrations, and VEGF-A concentration in cultured medium was not significantly decreased (our unpublished observation). Our preliminary data suggest that thyroid follicles cultured with high iodide concentrations produce humoral factors which inhibit proliferation of human umbilical venous endothelial cells. To elucidate which genes are regulated by iodide, we used a quantitative fluorescent cDNA microarray [55]. Fluorescent-labeled cDNA prepared from human thyroid follicles cultured in serum-free medium containing low (10^{-8} M) and high (10^{-5} M) iodide was hybridized and analyzed by a cDNA microarray. Among 2400 genes, expression levels of DNA dou-

Fig. 10. List of angiogenesis factors and inhibitors and possible mechanism of iodide-induced decrease in blood flow in Graves' thyroid glands.

bled in more than 100 genes. We are currently identifying which angiogenesis factors and/or inhibitors are involved in the iodide-induced decrease in thyroid blood flow.

Summary

Using thyroid follicles in suspension culture, in which thyroid function is maintained for a couple of weeks, while preserving Wolff-Chaikoff effect and responding to physiological concentrations of TSH ($0.1 \mu\text{U/ml}$), we have partly elucidated the pathophysiology of increased blood flow in Graves' thyroid gland. Furthermore, we are investigating the mechanism by which iodide inhibits thyroid blood flow.

Since administration of a large dose of iodide prior to subtotal thyroidectomy decreases thyroid blood flow and reduces operative morbidity and mortality in patients with Graves' disease, it is important to elucidate the mechanism by which thyroid blood flow is regulated. Furthermore, this research field will be clinically important to develop new strategies for the treatment of hypervascular and aggressive thyroid carcinoma, particularly anaplastic thyroid carcinoma. A recent report that anti-VEGF antibody re-

duced cancer growth in nude mice transplanted with human thyroid carcinoma is an indication this field holds for future studies [56].

Acknowledgments

This work was presented as the Honorary Lecture for the Fourth Annual Meeting of the Japan Endocrine Pathology Society, on November 11, 2000, at the Tokyo Women's Medical University (TWMU). We thank the following researchers and doctors for their roles in this work. Yamazaki K, Yamada E, Kanaji Y (Thyroid Disease Institute, Kanaji Hospital), Shizume K (Professor Emeritus of TWMU, former President of Growth Foundation in Japan), Kato Y, Kobayashi M (Department of Pathology, TWMU), Nakajima K, Miyakawa M, Takano K (Department of Medicine, Institute of Clinical Endocrinology, TWMU), Fellmer P, Obara T (Department of Surgery, Institute of Clinical Endocrinology, TWMU), Shibuya M (Division of Genetics, Institute of Medical Science, University of Tokyo). The author is particularly grateful to Professors Takano K, Kobayashi M, Obara T, and Shibuya M, for their critical review of the manuscript.

References

1. Ferrara N (2001) Role of vascular endothelial growth factor in regulation of physiological angiogenesis. *Am J Physiol Cell Physiol* 280: C1358–1366.
2. Ferrara N (2000) Vascular endothelial growth factor and the regulation of angiogenesis. *Recent Prog Horm Res* 55: 15–36.
3. Ralls PW, Mayekawa DS, Lee KP, Colletti PM, Boswell WD, Halls JM (1988) Color-flow Doppler sonography in Graves' disease: "thyroid inferno". *Am J Roentgenol* 150: 781–784.
4. Sato K, Miyakawa M, Eto M, Inaba T, Matsuda N, Shiga T, Ohnishi S, Kasanuki H (1999) Clinical characteristics of amiodarone-induced thyrotoxicosis and hypothyroidism in Japan. *Endocr J* 46: 443–451.
5. Wollman SH, Hervig JP, Zelig JD, Ericson LE (1978) Blood capillary enlargement during the development of thyroid hyperplasia in the rat. *Endocrinology* 102: 2306–2314.
6. Zeligs JD, Wollman SH (1979) Mitosis in thyroid follicular epithelial cells in vivo. III. Cytokinesis. *J Ultrastruct Res* 66: 288–303.
7. Ericson LE, Wollman SH (1980) Ultrastructural aspects of capillary fusion during the development of thyroid hyperplasia. *J Ultrastruct Res* 72: 300–315.
8. Shibuya M, Ito M, Claesson-Welsh L (1999) Structure and function of vascular endothelial growth factor receptor-1 and -2. In: *Vascular Growth Factors and Angiogenesis*. eds. Claesson-Welsh L, 1–30, Springer-Verlag.
9. Nitsch L, Wollman SH (1980) Suspension culture of separated follicles consisting of differentiated thyroid epithelial cells. *Proc Natl Acad Sci USA* 77: 472–476.
10. Sato K, Satoh T, Shizume K, Ozawa M, Han D, Imamura H, Tsushima T, Demura H, Kanaji Y Jr, Ito Y, Obara T, Fujimoto Y, Kanaji Y (1990) Inhibition of ^{125}I organification and thyroid hormone release by interleukin-1, tumor necrosis factor- α , interferon- γ in human thyrocytes in suspension culture. *J Clin Endocrinol Metab* 70: 1735–1743.
11. Yamazaki K, Sato K, Shizume K, Kanaji Y, Ito Y,

- Obara T, Nakagawa T, Koizumi T, Nishimura R (1995) Potent thyrotropic activity of human chorionic gonadotropin variants in terms of ^{125}I incorporation and *de novo*-synthesized thyroid hormone release in human thyroid follicles. *J Clin Endocrinol Metab* 80: 473–479.
12. Yamazaki K, Yamada E, Kanaji Y, Shizume K, Wang D, Maruo N, Obara T, Sato K (1996) Interleukin-6 (IL-6) inhibits thyroid function in the presence of soluble IL-6 receptor in cultured human thyroid follicles. *Endocrinology* 137: 4857–4863.
13. Sato K, Yamazaki K, Shizume K, Kanaji Y, Obara T, Ohsumi K, Demura H, Yamaguchi S, Shibuya M (1995) Stimulation of thyroid stimulating hormone and Graves' immunoglobulin G of vascular endothelial growth factor mRNA expression *in vitro* and *flt* mRNA expression in the rat thyroid *in vivo*. *J Clin Invest* 96: 1295–1302.
14. Viglietto G, Romano A, Manzo G, Chiappetta G, Paoletti I, Califano D, Galati MG, Mauriello V, Bruni P, Lago CT, Fusco A, Persico MG (1997) Up-regulation of the angiogenic factors PlGF, VEGF and their receptors (Flt-1, Flk-1/KDR) by TSH in cultured thyrocytes and in the thyroid gland of thiouracil-fed rats suggest a TSH-dependent paracrine mechanism for goiter hypervascularization. *Oncogene* 15: 2687–2698.
15. Nagura S, Katoh R, Miyagi E, Shibuya M, Kawaoi A (2001) Expression of vascular endothelial growth factor (VEGF) and VEGF receptor-1 (Flt-1) in Graves disease possibly correlated with increased vascular density. *Hum Pathol* 32: 10–17.
16. Larsen PR, Ingbar SH. The thyroid gland. In: Wilson JD, Foster DW (eds) Williams Textbook of Endocrinology, the 8th edition, W.B Saunders Company, Philadelphia, p. 357–487, 1992.
17. Soh EY, Sobhi SA, Wong MG, Meng YG, Siperstein AE, Clark OH, Duh QY (1996) Thyroid-stimulating hormone promotes the secretion of vascular endothelial growth factor in thyroid cancer cell lines. *Surgery* 120: 944–947.
18. Iitaka M, Miura S, Yamanaka K, Kawasaki S, Kitahama S, Kawakami Y, Kakinuma S, Oosuga I, Wada S, Katayama S (1998) Increased serum vascular endothelial growth factor levels and intrathyroidal vascular area in patients with Graves' disease and Hashimoto's thyroiditis. *J Clin Endocrinol Metab* 83: 3908–3912.
19. Ryuto M, Ono M, Izumi H, Yoshida S, Weich HA, Kohno K, Kuwano M (1996) Induction of vascular endothelial growth factor by tumor necrosis factor alpha in human glioma cells. Possible roles of SP-1. *J Biol Chem* 271: 28220–28228.
20. Ajjan RA, Watson PF, McIntosh RS, Weetman AP (1996) Intrathyroidal cytokine gene expression in Hashimoto's thyroiditis. *Clin Exp Immunol* 105: 523–528.
21. Klein M, Picard E, Vignaud JM, Marie B, Bresler L, Toussaint B, Weryha G, Duprez A, Leclere J (1999) Vascular endothelial growth factor gene and protein: strong expression in thyroiditis and thyroid carcinoma. *J Endocrinol* 161: 41–49.
22. Ramsden JD, Cocks HC, Shams M, Nijjar S, Watkinson JC, Sheppard MC, Ahmed A, Eggo MC (2001) Tie-2 is expressed on thyroid follicular cells, is increased in goiter, and is regulated by thyrotropin through cyclic adenosine 3',5'-monophosphate. *J Clin Endocrinol Metab* 86: 2709–2716.
23. Ramsden JD (2000) Angiogenesis in the thyroid gland. *J Endocrinol* 166: 475–480.
24. Sato K, Miyakawa M, Onoda N, Demura H, Yamashita T, Miura M, Kasajima T, Yamazaki K, Obara T (1997) Increased concentration of vascular endothelial growth factor/vascular permeability factor in cyst fluid of enlarging and recurrent thyroid nodules. *J Clin Endocrinol Metab* 82: 1968–1973.
25. Viglietto G, Maglione D, Rambaldi M, Cerutti J, Romano A, Trapasso F, Fedele M, Ippolito P, Chiappetta G, Botti G (1995) Upregulation of vascular endothelial growth factor (VEGF) and downregulation of placenta growth factor (PlGF) associated with malignancy in human thyroid tumors and cell lines. *Oncogene* 11: 1569–1579.
26. Soh EY, Duh QY, Sobhi SA, Young DM, Epstein HD, Wong MG, Garcia YK, Min YD, Grossman RF, Siperstein AE, Clark OH (1997) Vascular endothelial growth factor expression is higher in differentiated thyroid cancer than in normal or benign thyroid. *J Clin Endocrinol Metab* 82: 3741–3747.
27. Bunone G, Vigneri P, Mariani L, Buto S, Collini P, Pilotti S, Pierotti MA, Bongarzone I (1999) Expression of angiogenesis stimulators and inhibitors in human thyroid tumors and correlation with clinical pathological features. *Am J Pathol* 155: 1967–1976.
28. Fenton C, Patel A, Dinauer C, Robie DK, Tuttle RM, Francis GL (2000) The expression of vascular endothelial growth factor and the type 1 vascular endothelial growth factor receptor correlate with the size of papillary thyroid carcinoma in children and young adults. *Thyroid* 10: 349–357.
29. Klein M, Vignaud JM, Hennequin V, Toussaint B, Bresler L, Plenat F, Leclere J, Duprez A, Weryha G (2001) Increased expression of the vascular endothelial growth factor is a pejorative prognosis marker in papillary thyroid carcinoma. *J Clin Endocrinol Metab* 86: 656–658.
30. Lennard CM, Patel A, Wilson J, Reinhardt B, Tuman C, Fenton C, Blair E, Francis GL, Tuttle RM (2001) Intensity of vascular endothelial growth factor expression is associated with increased risk of recurrence and decreased disease-free survival in papillary thyroid

- cancer. *Surgery* 129: 552–558.
31. LiVolsi VA. Pathology. In: Braverman LE, Utiger RD (eds) *Werner and Ingbar's the Thyroid: A Fundamental and Clinical Text*, p. 497–520, 7th edition, Lippencott-Raven, New York, 1992.
 32. Karkkainen MJ, Petrova TV (2000) Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis. *Oncogene* 19: 5598–5605.
 31. Li X, Eriksson U (2001) Novel VEGF family members: VEGF-B, VEGF-C and VEGF-D. *Int J Biochem Cell Biol* 33: 421–426.
 34. Fellmer PT, Sato K, Tanaka R, Okamoto T, Kato Y, Kobayashi M, Shibuya M, Obara T (1999) Vascular endothelial growth factor-C gene expression in papillary and follicular thyroid carcinoma. *Surgery* 126: 1056–1062.
 35. Shushanov S, Bronstein M, Adelaide J, Jussila L, Tchipysheva T, Jacquemier J, Stavrovskaya A, Birnbaum D, Karamysheva A (2000) VEGF_C and VEGFR3 expression in human thyroid pathologies. *Int J Cancer* 86: 47–52.
 36. Karpanen T, Egeblad M, Karkkainen MJ, Kubo H, Yla-Herttuala S, Jaattela M, Alitalo K (2001) Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. *Cancer Res* 61: 1786–1790.
 37. Mandriota SJ, Jussila L, Jeltsch M, Compagni A, Baetens D, Prevo R, Banerji S, Huarte J, Montesano R, Jackson DG, Orci L, Alitalo K, Christofori G, Pepper MS (2001) Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO J* 20: 672–682.
 38. Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, Riccardi L, Alitalo K, Claffey K, Detmar M (2001) Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med* 7: 192–198.
 39. Yonemura Y, Endo Y, Fujita H, Fushida S, Ninomiya I, Bandou E, Taniguchi K, Miwa K, Ohoyama S, Sugiyama K, Sasaki T (1999) Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer. *Clin Cancer Res* 5: 1823–1829.
 40. Tsurusaki T, Kanda S, Sakai H, Kanetake H, Saito Y, Alitalo K, Koji T (1999) Vascular endothelial growth factor-C expression in human prostatic carcinoma and its relationship to lymph node metastasis. *Br J Cancer* 80: 309–313.
 41. Niki T, Iba S, Tokunou M, Yamada T, Matsuno Y, Hirohashi S (2000) Expression of vascular endothelial growth factors A, B, C, and D and their relationships to lymph node status in lung adenocarcinoma. *Clin Cancer Res* 6: 2431–2439.
 42. Hashimoto I, Kodama J, Seki N, Hongo A, Yoshino-uchi M, Okuda H, Kudo T (2001) Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO J* 20: 672–682.
 43. O-charoenrat P, Rhys-Evans P, Eccles SA (2001) Expression of vascular endothelial growth factor family members in head and neck squamous cell carcinoma correlates with lymph node metastasis. *Cancer* 92: 556–568.
 44. Kabashima A, Maehara Y, Kakeji Y, Sugimachi K (2001) Overexpression of vascular endothelial growth factor C is related to lymphogenous metastasis in early gastric carcinoma. *Oncology* 60: 146–150.
 45. Cao Y, Linden P, Farnebo J, Cao R, Eriksson A, Kumar V, Qi JH, Claesson-Welsh L, Alitalo K (1998) Vascular endothelial growth factor C induces angiogenesis in vivo. *Proc Natl Acad Sci USA* 95: 14389–14394.
 46. Ristimaki A, Narko K, Enholm B, Joukov V, Alitalo K (1998) Proinflammatory cytokines regulate expression of the lymphatic endothelial mitogen vascular endothelial growth factor-C. *J Biol Chem* 273: 8413–8418.
 47. Becks GP, Eggo MC, Burrow GN (1987) Regulation of differentiated thyroid function by iodide: preferential inhibitory effect of excess iodide on thyroid hormone secretion in sheep thyroid cell cultures. *Endocrinology* 120: 2569–2575.
 48. Nakajima K, Yamazaki K, Yamada E, Kanaji Y, Kosaka S, Sato K, Takano K (2001) Amiodarone stimulates interleukin-6 production in cultured human thyrocytes, exerting cytotoxic effects on thyroid follicles in suspension culture. *Thyroid* 11: 101–109.
 49. Wolff J (2001) Physiology and pharmacology of iodized oil in goiter prophylaxis. *Medicine (Baltimore)* 80: 20–36.
 50. Yuasa R, Eggo MC, Meinkoth J, Dillmann WH, Burrow GN (1992) Iodide induces transforming growth factor beta 1 (TGF-beta 1) mRNA in sheep thyroid cells. *Thyroid* 2: 141–145.
 51. Gartner R, Schopohl D, Schaefer S, Dugrillon A, Erdmann A, Toda S, Bechtner G (1997) Regulation of transforming growth factor beta 1 messenger ribonucleic acid expression in porcine thyroid follicles in vitro by growth factors, iodine, or delta-iodolactone. *Thyroid* 7: 633–640.
 52. Eng PHK, Cardona GR, Fang SL, Previti M, Alex S, Carrasco N, Chin WW, Braverman LE (1999) Escape from the acute Wolff-Chaikoff effect is associated with a decrease in thyroid sodium/iodide symporter messenger ribonucleic acid and protein. *Endocrinology* 140: 3404–3410.
 53. Chang DC, Wheeler MH, Woodcock JP, Curley I, Lazarus JR, Fung H, John R, Hall R, McGregor AM (1987) The effect of preoperative Lugol's iodine on thyroid blood flow in patients with Graves' hyper-

- thyroidism. *Surgery* 102: 1055–1061.
54. Armtznus AB, Smit LJ, Schipper J, van der Heide, Meinders AE (1991) Inverse relation between iodine intake and thyroid blood flow: color Doppler flow imaging in euthyroid humans. *J Clin Endocrinol Metab* 73: 1051–1055.
55. Yamazaki K, Yamada E, Kanaji Y, Sato K, Takano K, Obara T (2001) Genes regulated by TSH and iodide in cultured human thyroid follicles: analysis by cDNA microarray. The 73rd Meeting of the American Thyroid Association, September 10–14, Maryland, Abstract #20.
56. Soh EY, Eigelberger MS, Kim KJ, Wong MG, Young DM, Clark OH, Duh QY (2000) Neutralizing vascular endothelial growth factor activity inhibits thyroid cancer growth in vivo. *Surgery* 128: 1059–1065.
57. Sato K, Yamazaki K, Yamada E, Kanaji Y, Miura M, Obara T (1999) Immunoglobulin G of untreated Graves' patients with or without TSH receptor antibody (determined by porcine thyrocytes) universally elicit potent thyroid hormone-releasing activity in cultured human thyroid follicles. *Thyroid* 9: 979–988.