I. GENERAL CONSIDERATIONS OF THYROID HORMONE SYNTHESIS

The thyroid gland is an organ belonging to the endocrine system. Therefore, the primary function of the thyroid is the formation, storage, and secretion of thyroid hormones, such as thyroxine (T4) and triiodothyronine (T3). Thyroid hormone formation involves a coordinated series of steps controlled by thyrotropin (TSH). This includes thyroglobulin (TG) synthesis and vectorial transport to the lumen of the thyroid follicles making up the gland, where TG is stored. A concentrative iodine uptake by the sodium iodine transporter (NIS), as well as iodination of TG and coupling of TG iodothyronine residues by the thyroid peroxidase (TPO) were reported. In recent studies, it has been demonstrated that expression of the TG, TPO, NIS, and TSH receptor (TSHR) genes is regulated by thyroid-restricted transcription factors such as thyroid transcription factor 1 (TTF-1) and 2 (TTF-1 and TTF-2), and PAX8 (Fig. 1). All of them bind to TG and TPO promoters. TTF-1 and PAX8, however, preferentially bind to the TG and TPO promoters, respectively. In addition, TTF-1 binds to the TSHR and NIS gene promoters. The role of TTF-1 as a critical transcription factor regulating the gene expression of thyroid-specific proteins has been well documented in cell culture systems, particularly the FRTL-5 cell line. However, there has been very little information on the distribution and localization of TTF-1 in human thyroid tissues. Interestingly, it was reported by Suzuki et al. that follicular TG suppresses the expres-
sion of vascular endothelial growth factor (VEGF/VPF) expression as well as NIS expression. Miyagi et al. more recently, reported that thyroid stimulating hormone (TSH) down-regulates VEGF expression in FRTL-5 cells. Therefore, it may be possible that there is a close relation between thyroid hormone production and vascular permeability.

Despite the fact that each follicle has a uniform TSH supply and reasonably uniform TSHR expression, thyroid follicles are quite heterogeneous not only in their shape, size or colloid density, but also in their functions. Heterogeneity in protein expression and cell growth exists certainly even in diseased thyroid. During quantitative immunoelectron and immunohistochemical analysis of TG synthesis under various experimental conditions, we noted that TG synthesis and secretion into the follicular lumen were regulated by an unknown constitutive mechanism and not by TSH. Suzuki et al. suggested that TG protein, a major component of the colloid, or something within the colloid, or something within colloid, might act on follicular functions as an autocrine regulator because degradation of colloid appeared to correlate with TTF-1 expression and TG synthesis. In fact, previous studies using FRTL-5 rat thyroid cells may support this hypothesis; TG is a feedback suppressor of TTF-1, PAX8, or TTF-2 gene expression and regulation of these thyroid-specific transcription factors appears to be specific, which results in decreased TSHR, NIS, and TPO, as well as TG gene expression. In thyroid follicles, it can be suggested that the accumulation of TG within the follicular lumen can decrease follicular function by initiating this feedback. TG acts an autocrine/paracrine regulator within a single follicle; it may contribute to follicular heterogeneity by limiting individual follicle size, cell growth, and cell function.

Fig. 1. Structure of thyroglobulin (TG), thyroperoxidase (TPO) and thyrotropin receptor (TSHR) promoters. Symbols refer to binding sites mapped by DNAse I footprinting. The structure of these promoters is drawn from finding of no.18.
II. EXPRESSION OF TTF-1 IN ORGANS

TTF-1 was cloned as a transcription factor important for thyroid-specific and maximal expression of TG19). Subsequent studies showed that TTF-1 was also important for thyroid-specific and maximal expression of TPO, TSHR, and NIS genes4–9). TTF-1 functions together with PAX8 that binds to a sequence overlapping one of the TTF-1 recognition sites. TTF-1 protein has been detected very early during thyroid development: 5 days before the appearance of TG and TPO mRNA20). It has, therefore, been proposed that TTF-1 may also have a role in thyroid cell commitment. A knockout study established that TTF-1 is essential for the organogenesis of the lung, ventral forebrain, and pituitary, as well as the thyroid21). Therefore, TTF-1 is not thyroid-specific but thyroid-restricted. The present Northern blot analysis showed that expression of TTF-1 mRNA was clearly demonstrated in the lungs as well as thyroid (Fig. 2). TTF-1 mRNA expression was also evident with RT-PCR analysis in the brain and pituitary as well as these organs (Fig. 3). In the lung, TTF-1 is a transcription factor important in the expression of surfactants22,23); the role of TTF-1 as a transcription factor regulating neural function is still unknown.

III. EXPRESSION OF TTF-1 IN NORMAL AND NEOPLASTIC THYROID TISSUES

Little is known about the localization and distribution of TTF-1 proteins and mRNA in normal thyroid tissues, even though many studies on TTF-1 have been reported in the past 10 years24). The present immunohistochemical study with antibody for TTF-1 demonstrated a nuclear localization of TTF-1 protein in most follicular cells of human thyroids25,26). On ISH (in situ hybridization) observations, an antisense TTF-1 probe but not a counterpart sense probe exhibited a positive signal for TTF-1 mRNA in the perinuclear region of the thyroid follicular epithelium9). Expression levels of TTF-1 mRNA among follicles were not uniform in normal thyroid25)(Fig. 4). Slight expression of TTF-1 mRNA was detected in the flat thyroid epithelium lining the lumen of large follicles.

![Northern blotting showing TTF-1 mRNA expression in rat lung (lane 5) and thyroid (lane 10).](image)
contrast, the cuboidal or columnar cells of small follicles showed strong expression of TTF-1 mRNA. Immunohistochemical positivity for TG or TPO was also variable among follicles. These findings may suggest "functional follicular heterogeneity" of the thyroid.

The production of TG, T4, and T3 was frequently evident with the immunoperoxidase methods in nontoxic thyroid tumors\(^ {27}\). Recently, Saito et al.\(^ {28}\) reported an increased expression of the sodium/iodine symporter in papillary thyroid carcinomas. The appearance of oxalate crystals in the follicular lumen of tumors is associated with follicular function estimated by immunohistochemistry\(^ {29,30}\). Fabbro et al.\(^ {31}\) investigated TTF-1 mRNA expression in thyroid neoplasms by Northern blotting and demonstrated that TTF-1 mRNA is well detectable in all human follicular cell tumors except undifferentiated carcinoma. The present study also showed TTF-1 mRNA expression to varying degrees in thyroid tumors as well as normal thyroids\(^ {25,26}\) (Fig. 5).

By immunoperoxidase staining with anti-TTF-1 antibody, normal thyroid cells exhibit positive reaction products in the nuclei of most follicular cells as a fine granular pattern\(^ {25}\). A strong nuclear positivity of TTF-1 protein was also observed in all differentiated follicular cell tumors\(^ {25}\) (Fig. 6). Therefore, it could be suggest-

![TTF-1 and Beta-actin images](image)

**Fig. 3.** RT-PCR analysis of TTF-1 mRNA. All specimens showing TTF-1 mRNA expression in rat brain (lane 1) and pituitary (lane 4) as well as lung (lane 6) and thyroid (lane 5).

![ISH image](image)

**Fig. 4.** ISH (in situ hybridization) demonstrating expression of TTF-1 mRNA in normal follicular cells are not uniform among follicles.
ed that immunohistochemistry for TTF-1 facilitates defining the spectrum of thyroid tumors, and also is useful for the distinction between tumors of different histogenesis.32)

IV. TTF-1 IN C CELLS AND MEDULLARY CARCINOMA

Thyroid epithelial cells can be classified into 2 types according to embryonic origin, those of foregut endoderm (follicular cells) and those of neuroectodermal deviation (C cells) origin. C cells are not common in adult human thyroid and, therefore, it is quite difficult to observe human C cells by electronmicroscopy.33) Medullary thyroid carcinoma originates from thyroid C cells34). This type of thyroid cancer is histologically characterized by a solid growth pattern, amyloid deposits in the intercellular spaces and stroma, and by production of large amounts of calcitonin. In the course of in situ hybridization studies examining the role of TTF-1 in thyroid function in adult rat thyroid in vivo, we identified TTF-1 mRNA in C cells and in parathyroid cells.35) Furthermore, Suzuki et al.36) hypothesized that TTF-1 similarly coordinates Ca²⁺-dependent gene expression in all rat cells in which TTF-1 and the Ca²⁺-sensing receptor (CaSR) are expressed, that is, parathy-

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Fig. 5. Northern blotting showing that TTF-1 mRNA is well detectable in all human thyroid tumors (lanes 4 to 13) including medullary carcinoma.

Fig. 6. Immunohistochemistry for TTF-1 demonstrating a strong nuclear positivity of papillary carcinoma cells.
roid cells, neural cells in the anterior pituitary or hippocampus, and keratinocytes. The present Northern blot analysis clearly demonstrated the presence of TTF-1 mRNA in medullary carcinoma tissue as well as normal thyroid and follicular cell tumors20) (Fig. 5). This finding was supported by Northern blot observations in the medullary carcinoma cell line (Fig. 7). In addition, immunohistochemistry using anti-TTF-1 antibody and in situ hybridization using an antisense TTF-1 riboprobe revealed that normal C cells that showed calcitonin positivity were stained a greater proportion of cells displaying a rather weak and heterogeneous response, in comparison with follicular cells20).

V. ANGIOARCHITECTURE IN THYROID TUMORS

It has been suggested that neoplastic cells of the thyroid tend to create an environment conducive to their progression37). The vascular network can be of importance in hormone production as well as nutrition or growth of thyroid tissue because follicular TG and TSH regulate the expression of VEGF10). Therefore, it is worth investigating the vascular architectures of normal and neoplastic thyroid tissues. Scanning electron microscopy (SEM) with resin casts has been employed to investigate angioarchitectures in neoplastic tissues20). This method, however, has been used mainly in experimental animals and is difficult to apply to surgically removed human materials, because it requires the injection of resin into the tissue immediately before use. On the other hand, of the various basement membrane proteins, type IV collagen is the most abundant molecule and serves as the major structural framework39). Therefore, immunohistochemistry for type IV collagen can be a method suitable for the observation of the vascular structure in various pathological states of the thyroid39,41). We established a novel technique using confocal laser scanning microscopy (CLSM) combined with immunohistochemistry for type IV collagen that clearly visualizes 3-dimensional images of the microvascular structure42). On CLSM observation, normal thyroid follicles are tightly covered by branching vessels being regular in shape and show no significant variation in caliber. Although the angioarchitectures of follicular neoplasms such as follicular adenoma and follicular carcinoma are basically similar to that of normal thyroid follicles, the neoplastic microvessels are more prominent and more irregular. Interestingly, all microfollicular adenomas show that neoplastic small follicles are closely attached to the capillaries and have a "grape-like" appearance42) (Fig. 8). This feature is hardly recognized using conventional immunoperoxidase technique. In contrast to follicular neoplasms, microvessels in papillae of papillary carcinoma are occasionally well developed and show a digital appearance. Papillary carcinomas also exhibit dense accumulation of
irregularly arranged microvessels with obvious pathological features, so-called aggregated vascular complex (Fig. 9). This feature is found only in papillae of papillary carcinoma. Our findings suggest that the fundamental vascular pattern varies by histological tumor type and appears to correlate well with the growth pattern, suggesting interdependence between parenchyma and stroma characteristics of thyroid tumors.

VI. VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN THYROID TUMORS

Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen and angiogenic factor in vivo, first isolated as an endothelial mitogen from pituitary follicular cells. VEGF is also known as vascular permeability factor (VPF) because the accumulation of VEGF on the endothelium of tumor blood vessels has been associated with hyperpermeability of these vessels for macromolecules. The products of two genes, the \textit{fms}-like tyrosine kinase (\textit{flt}-1) and the kinase insert domain-containing gene (\textit{KDR}/\textit{Flt}-1), have been shown to be high-affinity VEGF receptors.

In the thyroid, Sato \textit{et al.} reported that VEGF mRNA expression is stimulated in primary follicular cultures derived from patients with Graves’ disease by thyroid stimulating hormone (TSH) and Graves’ immunoglobulin. G. Soh \textit{et al.} suggested that TSH stimulated the secretion of VEGF protein in thyroid cancer cell lines and primary cultures of thyroid cancer. We also previously suggested that hypervascularity in Graves’ disease tissues is related to up-regulated

Fig. 8. Confocal laser scanning microscopic observation using immunohistochemistry for type IV collagen showing a “grape-like” appearance of microvessels in microfollicular adenoma.

Fig. 9. Confocal laser scanning microscopic observation using immunohistochemistry for type IV collagen showing dense accumulation of irregularly arranged microvessels with obvious pathological features, resembling a “glomeruloid vascular structure”.
VEGF expression in hyperplastic follicles\(^{49}\). However, more recently it was suggested that TSH significantly decreased mRNA levels of VEGF in the FRTL-5 thyroid cell line. Most metabolic functions of TSH were suggested to be mediated by cAMP. The effects of camp using \((\text{Bu})_2\text{cAMP}\) were similar to those of TSH. Therefore, it is suggested that TSH down-regulates VEGF expression in FRTL-5 cells\(^{12}\). It is conceivable that the discrepancy between in vivo and in vitro is caused at least in part by the different experimental conditions used to determine the VEGF expression. Recently, it was reported that thyroglobulin regulates thyroid-specific gene expression, including VEGF\(^{10,50}\). They also reported that the effect of thyroglobulin was significantly stronger than that effect of TSH. Therefore, thyroglobulin stored within the follicular lumen may effect VEGF expression.

It was reported that VEGF expression levels in malignant tumors are often higher than in normal tissues or less malignant tumors\(^{51}\) (Fig. 10). Northern blot analysis of thyroid mRNA with the human VEGF cDNA probe showed that the expression level of VEGF mRNA tended to be higher in thyroid tumors than normal tissues\(^{52}\). In situ hybridization showed the expression of VEGF mRNA in non-neoplastic follicular cells and tumor cells, whereas immunohistochemical staining showed that VEGF protein was localized not only in these epithelial cells but also in endothelial cells of stromal vessels. These findings are consistent with those of previous studies that suggested VEGF was involved in a paracrine mechanism in the tumor\(^{53}\).

A central fibrovascular stalk covered by a neoplastic epithelial lining characterizes the papillae of papillary carcinoma\(^{57}\). Our previous study using a confocal laser scanning microscope revealed that papillae of papillary carcinoma exhibit dense accumulation of irregularly arranged microvessels. In an in situ hybridization study, expression of VEGF mRNA in the cells covering the papillary areas tended to be more intense than that in the follicle forming cells (Fig. 11). Identical localizations of VEGF protein were immunohistochemically observed. These findings suggest that vascular structures in papillae of papillary carcinoma tissues are associated with strong production of VEGF in carcinoma cells. In contrast, in many instances, the papillae were swollen by edema fluid or occupied by an abundant acellular hyaline material. VEGF enhanced the permeability of tumor blood vessels as a vascular permeability factor. This suggested that VEGF-induced enhancement of vascular permeability leads to the stromal edema of the papillae and subsequently to the deposition of a fibrin matrix.

Fig. 10. Northern blotting showing that the expression level of VEGF mRNA tends to be higher in thyroid tumors (lanes 3 to 6, follicular adenoma; lanes 7 to 11, papillary carcinoma) than normal tissues (lanes 1 and 2).
around existing vessels, which in turn serves as a substrate for developing tumor stroma and ingrowing capillaries\(^{52}\). In addition, *in situ* hybridization suggested that VEGF mRNA was intensely expressed in undifferentiated carcinoma cells immediately adjacent to necrotic foci, which agrees with findings of recent studies that reported enhanced VEGF expression in specific subpopulations of glioblastoma cells bordering areas of necrosis, a process known as palisading\(^{54}\). These results suggested that VEGF upregulation in undifferentiated thyroid carcinomas may be associated with tumor necrosis. Hypoxia is thought to be a major cause of tumor necrosis\(^{55}\).

**VII. Future of Thyroid Pathology**

Advances in technological methods and major conceptual breakthroughs are forming the foundation on which new thyroid pathology is rapidly being established. For the pathologist, some of the most powerful technical advances have been those that wed morphology to the disciplines of biochemistry, immunology, and molecular biology. Tools such as immunohistochemistry have already contributed substantially, and offered new objective criteria for diagnosis and classification of thyroid diseases. New developments in recombinant DNA technology have made it possible to probe the intact cell and tissue for specific gene expression at the DNA and RNA level, and thereby create a new dimension in thyroid pathology for the study of a structural-functional correlation at the molecular level.

It could be possible to suggest that pathologists should consider using new techniques in histological or cytological diagnosis to help understanding endocrine diseases.

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