

# Immunohistochemical Investigation of Angiogenesis Activity in Thyroid Gland under Hashimoto's Thyroiditis *versus* Diffuse Toxic Goiter

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## Abstract

**Aim of Study:** To reveal the difference in structural-functional activity between Hashimoto's thyroiditis (HT) and diffuse toxic goiter (DTG) based on microvessel density and the immunoeexpression of vascular endothelial growth factor (VEGF), CD34, and thyroid-stimulating hormone (TSH) in thyrocytes and endothelial cells.

**Materials and Methods:** The authors studied, pathologically and immunohistochemically, the expression of TSH, CD34 and VEGF in postsurgical thyroid specimens in 20 cases per each group (HT, DTG, and nodular goiter) as well as in normal tissues, all obtained from the National Institute of Endocrinology (Tbilisi). Guidelines of the Japanese Thyroid Society were used for the clinico-morphological classification of HT. The microvessel density was assessed in VEGF-positive areas using digital image analysis.

**Results and Conclusions:** No significant difference was obtained for the expression of the VEGF and CD34 between the studied groups. In contrast, the present study confirmed the significant difference in TSH expression among the lesions regardless of their biological behavior; and the results showed that only microvessel density and vascular surface area could truly differentiate the thyroid pathologies (HT and DTG) that are manifested by severe lymphoplasmocytic infiltration.

**Keywords:** Hashimoto's thyroiditis, diffuse toxic goiter, nodular goiter, angiogenesis, immunohistochemistry

## Introduction

Hashimoto's thyroiditis (HT) is one of the most common causes of hypothyroidism in Georgia with a regional and global incidence of 1.3% in a series of 5000 children aged 11-18 years. In adults, the HT frequency exhibits clear difference between male and female population (being 10-15 times higher in females than in males). According to the WHO data, the incidence of new cases varies between 0.3-1.5 per 1000 subjects of general population annually (1-3). The clinico-morphological subtypes of HT reported in the literature are different and often closely related to chronic lymphocytic thyroiditis and diffuse toxic goiter (DTG); moreover, the serum level of vascular endothelial growth factor (VEGF)

increases in both diseases during intense lymphocyte infiltration in thyroid parenchyma, forming a transitional hypo- and hyperthyroidism status (4, 5, 6). The authors have previously discussed the involvement of Hurthle cells in the pathogenesis of autoimmune thyroiditis (7); and the epithelial cells producing thyroglobulin take part in many benign processes in thyroid diseases including HT (8, 9).

In these aspects, VEGF represents a growth stimulator for endothelial cells and is capable of intensifying cell growth in both physiological and pathological conditions (10, 11). Angiogenesis plays an important role in the development of goiter-type changes in thyroid gland in terms of the proliferation of endothelial cells preceding hyperplasia of thyrocytes in follicles, which leads to increased levels of VEGF in serum and intrathyroid vascular area in patients with both toxic goiter and HT (12). Active study of the process of vascularization is important for the development of therapeutic methods in the management of autoimmune processes. CD34 constitutes one of the main factors provoking endothelial cell proliferation, not only in the nodular process, but also primarily in inflammation such as in autoimmune activity (13-15). Recent data have demonstrated significant differences in terms of thyroid-stimulating hormone (TSH) distribution and expression in functional and nonfunctional nodules due to TSH receptor gene mutation. The authors' previous studies have suggested that TSH receptor status in HT is featured a more varied and heterogeneous expression, ranging from moderate to highly positive (16).

The link between the disorganization of the architectonics of follicles and the infiltration of thyroid parenchyma with the presence of mononuclear hematopoietic cells in addition to Hurtle-cell metaplasias was observed consistently in the authors' studies. The significance of the abovementioned combination for the prognosis of hypothyroidism must be clarified, especially because the T4 blood level in such patients was observed to be low (17). This has been shown by the work of Caturegli *et al.* (3), Tsagareli and Gogiashvili (18), and Iwama *et al.* (19). With respect to the identification of the morphological and functional state of thyroglobulin in HT and lymphocytic thyroiditis associated with benign changes in the gland parenchyma, an understanding of how frequently the lymphocytic thyroiditis implies the involvement of Hurthle cells and their inclusion in the diagnosis of thyroiditis are particularly important. Great significance is given to the immunohistochemical data, because lymphocytic thyroiditis has not been described systematically due to the insufficiently accurate assessment criteria (7).

## Materials and Methods

### Patients

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The data files from the National Institute of Endocrinology (Tbilisi, Georgia), and the tissues and films of thyroid gland verified pathologically served as the study materials, including samples of 59 HT and 83 DTG as well as 10 normal tissues from forensic autopsy and 20 nodular goiter (euthyroidism) (NG) as comparative groups. All surgeries were performed between 2011 and 2014. For case selection, clinical data including the laboratory and ultrasonographic findings, medical history and surgical indications, were collected from referral notes submitted at the time of operation. The tissue slices were stained with hematoxylin and eosin. For the clinical diagnosis of HT, the guidelines of the Japanese Thyroid Society were used (20), and the diagnosis was confirmed pathologically by at least two independent investigators and a medical council board. The authors also used the histological features of HT as reported by Li *et al.* as a reference (21).

#### Immunohistochemical methods

Formalin-fixed paraffin-embedded thyroid tissue sections were examined immunohistochemically to detect the expression of the two endothelial markers (VEGF and CD34) and TSH. Based on the WHO classification system, the thyroid lesions were divided into three groups: HT, DTG; and NG, with 20 cases for each group. Ten cases of normal thyroid were used as a control. All sections were fixed on poly-L-Lysine-coated glass slides and prepared as follows: 1) deparaffinization, rehydration and incubation for 30 min in 3% H<sub>2</sub>O<sub>2</sub>; 2) immersion in phosphate-buffered saline (PBS) for 20 min; and 3) antigen retrieval in microwave (600 W) for 20 min, followed by cooling in citrate buffer (0.01M, pH 6.0).

Specimens were incubated with the primary antibodies for one hour at room temperature and then rinsed three times with PBS at room temperature. The secondary antibody was applied and the immune complex was identified by streptavidin peroxidase. The primary antibodies included 1) human recombinant polyclonal anti-VEGF-165 antibody with dilution 1:50 (BioGenex, USA); 2) ready-to-use anti-TSH polyclonal antibody (BioGenex); and 3) ready-to-use monoclonal anti-CD34 antibody (clone QBEnd/10, Novocastra, UK). The results of immunoreactivity were examined visually in the presence of 0.05% 3,3'-diaminobenzidine and hydrogen peroxide. The nuclei were counterstained with haematoxylin and the slices were covered with DPX. All procedures were carried out according to the manufacturer's instruction (BioGenex). Quantitative data of the TSH, VEGF and CD34 expression and the microvascular density were obtained using a Daffodil MCX-100 light microscope equipped with a Sony digital camera (Austria). The immunoreactivities for TSH, CD34 and VEGF were measured quantitatively in 100 areas from each group, expressed as percentage of positive cells in total cells, and scored as 0 = negative; 1 = 0.1-25%; 2 = 25.1-50%, 3 = 50.1-80%, and 4 = 80.1-100% (14). To avoid false positive and negative responses in TSH reaction, samples from follicular adenoma were used as a positive control (16).

Microvessels were counted semi-quantitatively using the methods described by Rzeszutko *et al.* (13), and by Rydlova *et al.* (14). Microvessel density was assessed on VEGF-positive visual areas regardless of the presence or absence of visible vascular lumen; therefore the positively VEGF-stained isolated endothelial cells of cell clusters were counted as individual microvessels. First, the visual areas with the highest density of microvessels ("hot vascular sites" or "hot spots") were selected at a low magnification (x40), avoiding sites of fibrosis or lymphoid infiltration. The visual field containing the maximum number of microvessels was then examined at a higher magnification (x200), and the microvessels

were counted again. The number of vessels was defined as a mean value in terms of average microvessel count/field as described previously (16).

#### Statistical analysis

Statistical analysis was performed using Microsoft Excel 7.0 to determine the statistical differences for the microvessel density between the two groups of HT and DTG, with  $P < 0.05$  being considered statistically significant.

## Results and Discussion

#### Pathological study

HT was characterized by strong lymphoplasmocytic infiltration into the intrafollicular spaces, lymphoid follicle formation with enlarged germinal centers, dense stromal fibrosis, and reduced thyroid parenchyma. The thyroid follicles were reduced and replaced by lymphoid tissue. Sclerosis or hypervascularization was observed in some cases (Fig. 1a). Importantly, some cases of HT demonstrated the features of the fibrosis variant, with mild fibrosis in the stroma and predominant interfollicular fibrosis (Fig. 1b). NG, clinically defined as euthyroidism, showed sites of monomorphic hyperplasia of thyrocytes with diffuse-insular type, dystrophic secondary changes in follicles and stroma. Proliferation of polygonal cells distributed freely throughout the intercellular matrix was also observed in the parafollicular domain (Fig. 1c). DTG was mainly characterized by prominent hyperplasia in the intra- and parafollicular domains and multifocal lymphoid infiltration with lymphoid follicle formation. In contrast to HT, reduction and involution of thyroid follicles was not demonstrated; while hyperemia and hypervascularization were commonly found (Fig. 1d).

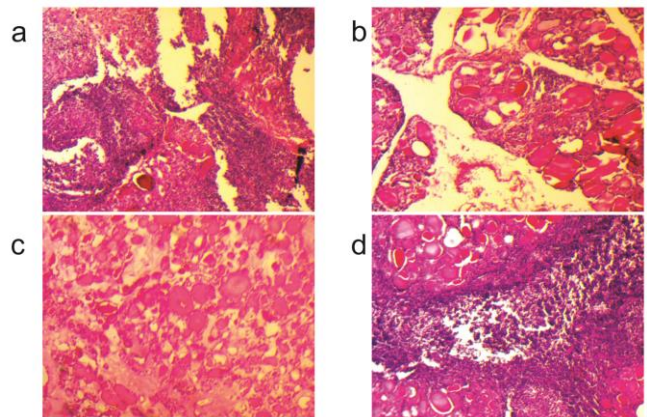


Fig. 1: Pathological patterns of thyroid lesions. 1a and 1b: Hashimoto's thyroiditis; 1c: nodular nontoxic goiter; and 1d: diffuse toxic goiter. Hematoxylin and eosin, x160

#### TSH expression

TSH expression was estimated in the follicular epithelial cells. In the NG, TSH was expressed weakly or moderately, showing 30% to 60% of thyrocytes being TSH-positive.

In HT, the TSH expression was different from that in NG. Diffuse lymphoplasmocytic infiltration with the formation of many lymphoid follicles as well as reduced and atrophic thyroid follicles were observed. More than 75% of the thyrocytes showed a negative or low TSH expression (Fig. 2).

Comparison of TSH expression in DTG revealed statistically significant difference ( $P = 0.001$ ) among the groups studied: within follicular components it was the diffuse type of TSH distribution, and diffuse stain for endothelial cells as well (Fig. 3).

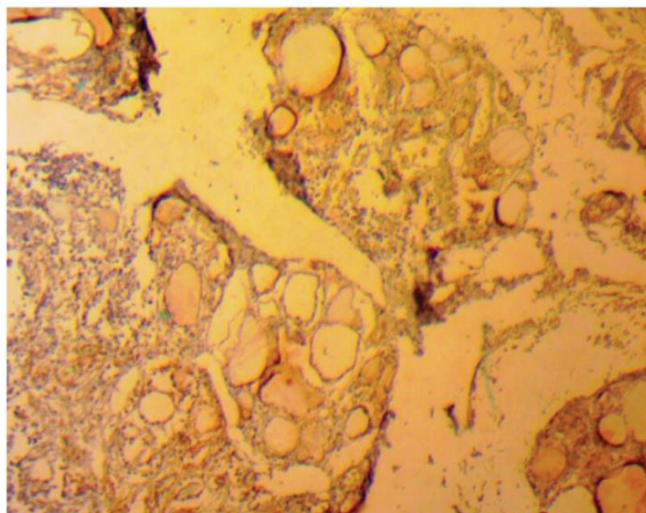


Fig. 2: Hashimoto's thyroiditis showing negative TSH expression with lymphocytic infiltration. Immunoperoxidase reaction (x160).

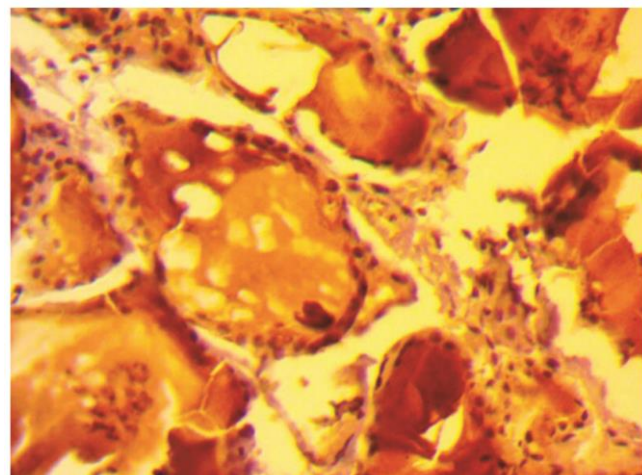


Fig. 3: Diffuse toxic goiter. Diffuse TSH expression in thyrocytes and endotheliocytes. Immunoperoxidase reaction (x200).

#### VEGF expression

VEGF activity in endothelial cells was detected in 18, 10 and 7 cases, respectively, out of each 20 cases of DTG, HT, and NG. Moreover, in the tested specimens focal cell expression as well as positivity in both endothelial and haemopoietic cells, especially in HT, were indicated. In the all cases of DTG, VEGF displayed elevated expression (>80% of cells) (Fig. 4a and 4b). No significant difference in the VEGF expression between NG and normal thyroid tissues was found in this study.

Expression of the endothelial cell marker CD34 was found in 16, 3, and 5 cases, respectively, out of each 20 cases of DTG, NG, and HT. Majority of the vessel walls and vessel microareas stained positive. Positive staining of cytoplasm of endothelial cells and thyrocytes was observed in DTG as well as in the normal tissues. In HT, positive signal was observed mainly in the cytoplasm of

endothelial cells (Fig. 5), whereas DTG had a diffuse expression (Fig. 6).

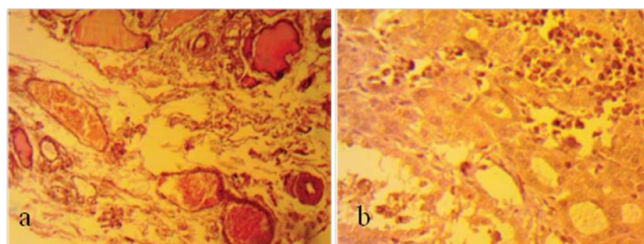


Fig. 4: a: Hashimoto's thyroiditis showing VEGF focal expression in endothelial cells; b: Diffuse toxic goiter showing VEGF high expression in follicular cells and endothelial membranes. Immunoperoxidase reaction (x160).

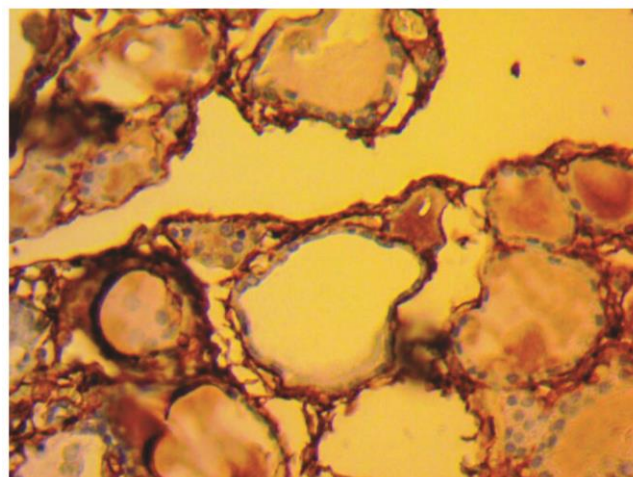


Fig.5: Hashimoto thyroiditis showing CD34 positive reaction in endotheliocytes (x200).

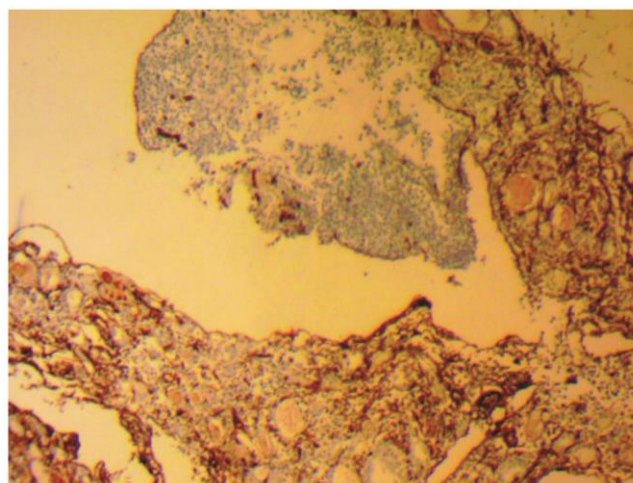


Fig. 6: Diffuse toxic goiter showing diffuse positive membranous CD34 staining. Immunoperoxidase reaction (x160).

The basal membrane of microvessels around follicles was found to be weakly VEGF-positive in all studied cases. The number of positive vessels was increased in DTG. The lowest density of positive microvessels was found in the tissue of NG, lower even than the control normal tissues.

### Assessment of MD

Microvessels were visualized by VEGF positivity and scored in three “hot spots” of each group studied. The MD value (range, mean, and standard deviation) indicated that angiogenesis and formation of new blood vessels occurred in DTG. Significant difference was observed between VEGF-positive vascular density between DTG and HT. No elevated expression of endothelial markers was found in either HT or NG (Table 1).

Table 1: Microvessel density and vascular area distribution

Thyroid pathology	Number of vessels (MVC)*		Vessel surface area (mm <sup>2</sup> )	
	Mean	SD	Mean	SD
	NG (n = 20)	30.3	12.5	0.278
DTG (n = 20)	39.0	12.3	0.393	0.105
HT (n = 20)	32	13.4	0.331	0.109

\*MVC, microvessel count

The authors' previous study, which focused on angiogenic potential and differentiation of thyroid lesions with different thyroid pathologies, indicated that the VEGF expression was present with the highest level in malignant tumors and multinodular goiter when compared with that in the normal tissues, which might be explained by the tumorigenic potential of multinodular goiter (13, 16, 18). Positive VEGF and TSH expression was observed in both thyrocytes and endothelial cells of microvessels of multinodular goiter, but lower than in HT.

The current study was designed to understand the activity of angiogenic factors in different thyroid pathologies accompanied by heavy lymphoplasmocytic infiltration and their potential implication as differential markers between DTG and HT. Despite a number of studies on thyroid angiogenesis showed that no endothelial antigens, including angiogenic regulators, CD34, and VEGF, are specific for either HT or DTG (12-14, 21), the present study demonstrated that the distribution patterns of CD34 and VEGF (focal or diffuse) were different between nodular nontoxic goiter (control) and DTG. Similar conclusion was reached for the MD, which was different between HT and DTG, two diseases with lymphoplasmocytic infiltration (12).

### Conclusions

Despite different pathological types of thyroid diseases, no marked difference was revealed in terms of the expression of VEGF and CD34 between HT and DTG in the present study. Only the MD and vascular surface area could differentiate the HT and DTG; both were manifested by severe lymphoplasmocytic infiltration patterns.

**Conflicts of Interest:** None

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