Vascular Endothelial Growth Factor – Marker for Proliferation in Thyroid Diseases?

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Abstract
Vascular endothelial growth factor (VEGF) is a critical regulator of angiogenesis and is involved in tumor development. To date, the role of VEGF in benign diseases of the thyroid is not well known. The purpose of the present study is to determine the expression of VEGF and its receptors in primary cultures of human thyrocytes.

Methods: 50 patients with uninodular (n = 11), multinodular (n = 15), recurrent goiter (n = 14) and Graves’ disease (n = 10) were enrolled. Nodular and corresponding paranodular tissue was obtained after surgery and investigated. RNA and protein were extracted from primary thyrocyte cultures. PCR, western blot and ELISA were performed to evaluate VEGF isoforms and VEGF receptor 1 and 2.

Results: Significantly increased transcription and protein expression of VEGF and its receptors were detected in nodular tissue of uninodular and recurrent goiter compared to the corresponding normal tissue. Active secretion of VEGF by thyrocytes was confirmed by ELISA. In multinodular goiter, no difference could be found between nodular and corresponding paranodular tissue in terms of expression of VEGF or its receptors. Furthermore, we found the highest levels of VEGF and its receptors in tissue obtained from patients with Graves’ disease.

Conclusion: Increased expression of VEGF and its receptors might be crucial in the proliferation of thyrocytes and therefore may contribute to the development of goiter and goiter recurrence.

Introduction
In 1989 vascular endothelial growth factor (VEGF) was identified as an angiogenic factor [1]. VEGF is a potent stimulator of endothelial cell growth and a critical regulator of both physiological and pathological angiogenesis [2]. VEGF binds to 2 different tyrosine kinase receptors VEGFR-1 (flt-1) and VEGFR-2 (flk-1/KDR), mainly expressed on endothelial cells [3]. VEGFR-1 may play a more regulatory role in angiogenesis, whereas VEGFR-2 is responsible for signaling [4]. In addition, VEGF mediates inflammatory reactions [5]. Via a paracrine mechanism VEGF signaling plays a critical role in tumor angiogenesis, hence, high expression of VEGF and its receptors was shown in a number of human cancers such as gastric cancer [6], breast cancer [7], colon cancer [8], ovarian cancer [9] and prostate cancer [10]. Increased expression of VEGF was even linked to poor outcome and increased risk for metastasis and tumor recurrence [11–14]. On the other hand, inhibition of VEGF, its receptors or signaling pathways leads to suppression of tumor angiogenesis and growth [15,16]. Several studies have demonstrated expression of VEGF and its receptors in benign and malignant thyroid tissue [17–19]. High expression of VEGF in thyroid cancer was found to be correlated with advanced tumor stage, lymph node metastasis and increased risk of recurrence [20–22]. Therefore, VEGF inhibitors are approved for the treatment of advanced thyroid cancers [23]. Furthermore, VEGF seems to be involved in goiter development by inducing endothelial cell proliferation and angiogenesis. Recently, in clinical studies it was demonstrated, that treatment with tyrosine kinase inhibitors leads to hypothyroidism in over 40% of patients, indicating a functional role of the VEGF signaling pathway even in normal thyroid tissue [24,25].
thyrocytes were plated at a density of 50,000 cells/well in 6-well plates and were cultured in DMEM supplemented with 10% fetal calf serum, 200 mM Hepes, 50 μM procollagen and 50 μg/ml streptomycin. The monocultures were maintained in a standard humidified incubator at 37°C in a 5% CO2 atmosphere. On day 1 medium was changed to remove cell debris, on day 4 harvesting was performed. Thyrocyte culture by this procedure has no detectable stromal or endothelial contamination, as attested to by immunohistochemistry and PCR.

**Immunofluorescence**

Primary thyrocyte cultures were washed with PBS (phosphate buffered saline) and fixed with ice cold acetone for 10 min. After fixation, cells were washed again followed by incubation with primary antibody for 60 min at 4°C. Primary antibodies were as follows: mouse monoclonal anti-cytokeratine 18 (clone Ks19.04) and 19 (clone Ks19.10) (Progen Biotechnik; Heidelberg, Germany), diluted 1:2; mouse monoclonal anti-fibroblast antibody (Acris antibodies; Hiddenshausen, Germany, diluted 1:100), mouse anti-vas Willebrand factor (Dako; Hamburg, Germany, diluted 1:50). After incubation with primary antibodies, cultures were washed twice with wash buffer (0.5 % BSA in PBS without Ca++ and Mg++) followed by incubation with secondary antibody for 60 min at 4°C (NL493 [NorthernLights] – conjugated donkey anti-mouse IgG, diluted to 1:100, (R&D Systems; Wiesbaden-Nordenstadt, Germany). All antibodies were diluted with 0.5 % BSA (bovine serum albumin) in PBS. Cell cultures were rinsed 3 times in wash buffer and finally mounted with Prolong antifade Kit (Molecular Probes; Eugene, Oregon). Staining was visualized microscopically by Zeiss Z-1 Observer (Zeiss; Göttingen, Germany). A negative control was also carried by incubating cell cultures with mouse IgG1 (Dako Cytomation, Denmark). All controls were consistently negative.

**Materials and Methods**

**Patients**

50 patients (♀ = 38, ♂ = 12) were enrolled in the clinical study. The protocol was approved by the ethics committee of the University Hospital of Frankfurt and preoperative written consent was obtained from all patients. The patients were divided into 4 groups according to the diagnosis, 11 patients with uninodeular goiter (UN), 15 with multinodular goiter (MN), 10 with Graves’ disease (G) and 14 patients with recurrent nodular goiter (R). All controls were consistently negative.

**Tissue samples and cell culture**

Thyroid tissue was obtained after surgical resection. All tissue samples were examined and characterized by a pathologist and when possible, nodular and adjacent non-tumor or paranodular tissues were acquired. In the case of Graves’ disease only paraneural nodules were investigated. The thyroid tissue was minced into small fragments, washed 3 times with HBSS (Hank’s balanced salt solution) containing 50 μM penciillin and 50 μg/ml streptomycin followed by enzymatic dissociation with 246 U/ml collagenase I for 60–90 min at 37°C. After digestion, thyrocytes were filtered through a 100μm nylon mesh (Becton Dickinson; Heidelberg, Germany), pelleted and washed with HBSS.

Thyrocytes were resuspended and counted in Neubauer counting chamber before plating. Cell viability was assessed using trypsin blue exclusion (>98%). Thyrocytes were plated at a density of 50,000 cells/well in 6-well plates and were cultured in DMEM supplemented with 10% fetal calf serum, 200 mM Hepes, 50 μM procollagen and 50 μg/ml streptomycin. The monocultures were maintained in a standard humidified incubator at 37°C in a 5% CO2 atmosphere. On day 1 medium was changed to remove cell debris, on day 4 harvesting was performed. Thyrocyte culture by this procedure has no detectable stromal or endothelial contamination, as attested to by immunohistochemistry and PCR.
analyzed by Gel Doc 100 with molecular analyst software (Bio-Rad Laboratories; Munich, Germany). Densitometric analysis was performed and signal was evaluated as area (mm²). GAPDH was used as housekeeping gene and a 100 bp DNA ladder was used as molecular weight marker. The results were confirmed by real-time quantitative PCR (qPCR) using RT² SYBR-Green/Rox qPCR master mix and gene specific primers (Table 2) (both from SA Bioscience, USA) according to the manufacturer’s protocol. Amplification of genes was performed using MX3005P (Stratagene, USA). 100 ng of cDNA was used in qPCR reaction in duplicates. The dissociation curve was checked at the end of each PCR reaction. GAPDH and β-Actin were used as housekeeping genes and for normalization. Gene expression (fold change) was calculated using ΔΔCt method after normalizing nodular tissue with each corresponding paranodular or normal adjacent tissue.

<table>
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<th>Target genes</th>
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<td>1222</td>
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Protein isolation and western blot
Thyrocytes cultures were rinsed twice with ice-cold PBS and lysed for 10 min on ice in lysis buffer (50 mM HEPES, 200 mM NaCl, 0.2 mM MgSO4, 0.4 mM phenylmethylsulfonyl fluoride, 2% Triton-X-100, 10 μg/mL leupeptine, 10 μg/mL aprotinine, 0.02% soybean trypsin inhibitor, 0.2 mM ortho- vanadate (Sigma-Aldrich; Taufkirchen, Munich, Germany). Cell lysates were centrifuged for 10 min at 12,000 × g at 4 °C. Protein concentration of the supernatants was determined by Coomassie Plus protein assay kit (Pierce; Rockford, IL, USA) and were measured spectrophotometrically at 595 nm by Tecan Infinite® M 200 microplate reader (Tecan-Deutschland; Crailsheim, Germany). Protein was denatured in Laemmli sample buffer (Bio-Rad Laboratories; Munich, Germany) with β-mercaptoethanol (Sigma; Taufkirchen, Germany), boiled for 5 min, transferred on ice and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (50 μg per lane). PeqGold prestained protein marker IV (Peqlab Biotechnologie GmbH; Erlangen, Germany) was used as molecular weight standards. After separation, protein was transferred to a polyvinylidene difluoride membrane (Hybond P; GE Healthcare; Munich, Germany). Blots were blocked with 10% low-fat milk for 1 h at room temperature followed by incubation with primary antibodies from Santa Cruz Biotechnology (rabbit polyclonal VEGF [total protein], 1:200; rabbit polyclonal VEGFR-1, 1:200; mouse polyclonal VEGFR-2, clone A-3, 1:200 and mouse monoclonal β Actin, clone AC-15, 1:1000; Sigma) overnight at 4 °C. Blots were then washed 3 × with Towbin buffer with 0.5% Tween 20 followed by incubation for 30 min with secondary antibody from Millipore GmbH; Schwalbach/Ts, Germany (polyclonal goat antirabbit IgG, 1:5000; goat anti-mouse IgG, 1:5000, both HRP conjugated) at room temperature. All antibodies were diluted in Towbin Buffer with 0.5% Tween 20 and 0.5% bovine serum albumin. Blots were then washed and incubated with ECL (Enhanced Chemiluminescence-ECL detection kit from GE Healthcare; Munich, Germany) and developed in Curix 60 (Agfa; Dusseldorf, Germany) and documented by Gel Doc 100. β Actin was used as loading control.

ELISA
Cell culture supernatant was quantified using VEGF ELISA-kit (R&D systems; Wiesbaden-Nordenstadt, Germany). The assay was done according to the manufacturer’s instructions. Briefly, 200 μl samples (1:30), standard, and controls were dispensed into microplate wells precoated with anti-VEGF monoclonal antibody, and then incubated for 120 min at room temperature. After incubation, the wells were washed 5 times and 50 μl of horse radish peroxidase (HRP) conjugated VEGF was added and incubated for 60 min at room temperature. After 5 washes, 100 μl of chromogenic enzyme substrate solution was added and incubated for 30 min at RT; 50 μl stop solution was added and spectrophotometrically measured using Infinite® M 200 microplate reader (Tecan-Deutschland; Crailsheim, Germany) at 405 nm. The sample concentration was calculated based on standard concentrations.

Statistical analysis
Data were analyzed using commercially available software Bias for Windows, Version 8.3.7 (H. Ackermann, Johann-Wolfgang Goethe University, Department of Mathematics). Data are presented as means ± SEM. Non-parametric tests were used due to irregularly distributed data. The groups were compared using Kruskal-Wallis test. Paired data (nodular and paranodular tissue) were analyzed using Wilcoxon’s test. Statistical significance was achieved at p < 0.05.

Results
Characterization of thyrocyte cultures
The primary cell cultures showed the typical morphology of thyrocytes on microscopic examination, i.e., small, elongated cells arranged as cellular isles (Fig. 1a, b). Immunohistochemical staining demonstrated the cells to be positive for cytokeratin 18 (Fig. 1c, d) and so to be of epithelial origin. The cells were negative for cytokeratin 19, including fibroblasts specific antigen and Factor VIII (von Willebrand factor), showing no contamination of stromal and endothelial cells, yielding primary thyrocyte culture purity of greater than 98%.

In addition, using RT-PCR all cell cultures were positive for thyroid peroxidase (TPO), TSH receptor (TSHR) and thyroglobulin (Tg), namely indicating the presence of thyrocytes (Fig. 1e).

mRNA expression of VEGF-A, VEGFR-1 and -2 in human thyrocytes
VEGF-A isoforms were expressed by 99% of all thyrocyte cultures. Here expression of VEGF145 was significantly higher in nodular tissue from uninodular goiter compared with corresponding normal tissue (VEGF145 = 70 ± 7 counts/mm² [UN] vs. 46 ± 7 counts/mm² [P]; p < 0.05) (Fig. 2a). In recurrent goiter we found higher amounts of VEGF145 mRNA in nodular tissue compared to paranodular tissue, but without statistical significance (Fig. 2c). VEGF-R1 and -R2 were significantly increased in nodular vs. paranodular tissue, as shown in uninodular goiter (VEGF-R1 = 479 ± 68 counts/mm² [UN] vs. 133 ± 47 counts/mm² [P]; p < 0.05; VEGF-R2 = 710 ± 72 counts/mm² [UN] vs. 361 ± 52 counts/mm² [P]; p < 0.05) (Fig. 2a) and recurrent...
goiter (VEGF-R1 = 428 ± 46 counts/mm² [UN] vs. 72 ± 20 counts/mm² [P]; p < 0.05; VEGF-R2 = 617 ± 56 counts/mm² [UN] vs. 174 ± 45 counts/mm² [P]; p < 0.05) (Fig. 2c).

In Graves’ disease we found the highest levels of VEGF145, VEGFR-1 and -2 mRNA compared to the paranodular tissue of the other 3 groups, but without reaching statistical significance (Fig. 2d).

Interestingly, in tissue from patients with multinodular goiter we did not find any significant difference in the expression levels of VEGF and its receptors between nodular and paranodular tissue (Fig. 2b).

The results of RT-PCR were confirmed by performing QPCR. The results are presented as fold change after normalizing nodular tissue with each corresponding paranodular tissue (Fig. 3a), as well as after normalizing paranodular tissue of Graves’ disease with paranodular tissue of the other 3 groups (Table 3b).

Protein expression of VEGF (total), VEGFR-1 and -2 in human thyrocytes

The VEGF protein was highly expressed in all cell cultures obtained. Furthermore, expression of the receptor proteins was demonstrated in over 90% of the isolated thyrocytes. In uninodular and recurrent goiter, protein expression of VEGF was higher in nodular compared to paranodular tissue as shown in Fig. 3a.

The same results were detected for protein expression of VEGFR-1 and VEGFR-2 (Fig. 3a).

Interestingly, again in multinodular goiter we could not find any difference in protein expression between paranodular and nodular tissue as seen at mRNA level (Fig. 3a).
In tissue from Graves’ disease we found the highest protein levels of VEGF, VEGFR-1 and -R2. Protein expression of VEGF and VEGFR-1 was significantly increased in comparison to the paranoalular tissue of the other 3 groups (VEGF = 0.7 ± [G] vs. 0.5 ± [UN]; 0.31 [MN]; 0.32 [R]; p < 0.05; VEGF-R1 = 0.7 ± [G] vs. 0.36 [UN]; 0.19 [MN]; 0.2 [R]; p < 0.05) (Fig. 3b).

Secretion of VEGF by human thyrocytes
All primary cell cultures secreted VEGF protein in the supernatant. The secretion level of VEGF was higher in nodular tissue of patients with recurrent goiter compared to paranoalular tissue, without reaching statistical significance (Fig. 4). Again, the highest levels of VEGF were measured in tissue obtained from Graves’ disease patients.

Discussion
VEGF, as an angiogenic factor, has been implicated in tumor angiogenesis and in progression of different malignant tumors. Therefore, it is important to investigate its role in thyroid cancer. So far, elevated VEGF expression seems to be associated with higher risk of metastasis, of recurrence and with a poor outcome in thyroid cancer [26]. To date, the expression of VEGF in normal thyroid tissue and its role in the development of goiter and Graves’ disease is not well determined.

In the present study, we were able to demonstrate co-expression of VEGF and its receptors VEGFR-1 and -2 in normal human thyrocytes and in benign diseases of the thyroid. In addition, we detected active secretion of VEGF protein by thyrocytes in the supernatant and measured higher concentrations of VEGF protein in nodular tissue of unino nodular and recurrent goiter compared to the corresponding normal tissue. Furthermore, the present data demonstrate large amount of VEGF protein in nodular and paranoalular tissue of multinodular goiter, that might contribute to the proliferating effect on thyrocytes and its involvement in goiter development.

These findings contradict previous studies, which reported weak expression of VEGF in benign nodules of the thyroid with no differences between normal thyroid tissue and benign adenomas [27,28]. Itoh et al. did not even find any expression of VEGF in normal thyroid tissue and only negative or weak expression in adenomatous goiter [29]. These discrepancies may be explained by the methodological approach used. In previous studies, fresh frozen tissue or formalin-fixed paraffin-embedded tissue was primarily used for the investigation while our study examined pure cultures of freshly isolated thyrocytes, thus, excluding possible endothelial contamination and antigen masking of targets of interest due to formalin-fixation.

With regards to the expression of the VEGF receptors, only immunohistochemistry staining of VEGF receptors expression on human thyrocytes of benign thyroid diseases has been shown...
Consistent with our data, Sato et al. reported high levels of VEGF in cyst fluid of thyroid nodules. They postulated that VEGF was secreted by thyrocytes and that it was involved in the pathogenesis of cyst fluid accumulation [31].

In the present study, we were able to detect an active secretion of VEGF by human thyrocytes and simultaneous expression of both VEGF receptors. This coexpression of VEGF and its receptors raises the possibility that VEGF may act in an autocrine loop in thyrocytes, as observed previously in thyroid cancer [32]. Perhaps, an autocrine stimulation plays a crucial role in proliferation of thyrocytes and such up-regulation of the VEGF signaling might be involved in the development of nodular goiter.

Besides, high levels of VEGF and its receptors in recurrent nodules may pose potential risk for the development of goiter recurrence.

It is also known, that autocrine stimulation of VEGF receptors leads to enhanced tumor growth [33]. This mechanism, initiated by increased VEGF expression, may also be involved in the growth of thyroid nodules and goiter recurrence.

In our study, we did not find any differences in gene or protein expression of VEGF and its receptors between nodular and para-nodular tissue of patients with multinodular goiter, but instead detected an increased concentrations of the growth factor in both tissues. Therefore, we postulated, that in multinodular goiter the entire tissue of the thyroid gland might be pathologically altered and are not only limited in the circumscribed nodules. Although the cause of recurrence might be of multifactorial origin, elevated expression of VEGF and its receptors might also contribute to the high rate of recurrence of over 20% of multinodular goiter after subtotal resection [34–36]. Our findings are also in accord with clinical studies which assumed an involvement of the whole thyroid parenchyma in benign disease and therefore, recommended total thyroidectomy as a suitable treatment [37].

Furthermore, we examined tissue from 10 patients with Graves’ disease. In the literature, contradictory data are reported on VEGF expression in this autoimmune disease of the thyroid gland. However, several authors could not detect any expression of VEGF in Graves’ disease in immunohistochemical staining [29] or only at lower levels compared to neoplastic tissue [30] and so argued that VEGF signaling plays only a minor role in the development of autoimmune disease of the thyroid gland. On the other hand, Iitaka et al. reported increased serum VEGF levels in patients with Graves’ disease compared to healthy persons [38]. Furthermore, studies demonstrated a positive correlation between VEGF levels and increased vascular density in Graves’ disease [39] as well as strong expression of VEGF in hyperplastic follicular cells and surrounding capillaries [40], indicating a link between epithelial function and microcirculation.

In concordance with these data, we have detected in our study high levels of VEGF and its receptors both in mRNA and protein in monocultures of thyrocytes obtained from patients with Graves’ disease. Here, we have shown that the expression was significantly increased compared with the other groups. These results may indicate that VEGF is involved in the pathogenesis of Graves’ disease and may have an influence on both epithelial proliferation and thyroid microcirculation, causing a typical hypervascularization and hyperplasia seen in this autoimmune disease. Moreover, this might be a first hint, that VEGF might also be involved in the control of thyroid function. Besides the up-regulation of VEGF in goiter, the present study was able to confirm the expression of VEGF and its receptors in

so far. Jebreel et al. reported no differences in VEGFR-1 and -2 expression between the different thyroid pathologies, so the author suggested that up-regulation of VEGF and not of its receptors was important in the development of thyroid diseases [30]. In contrast to our findings, we demonstrated significantly higher expression of the 2 receptors in nodular tissue of un-nodular and recurrent goiter compared to corresponding normal tissue both in mRNA and protein level. These results might indicate, that not only up-regulation of VEGF, but also up-regulation of its receptors plays a critical role in the development of goiter.

In addition to receptor expression, we were able to detect significant amount of VEGF in the supernatant of thyrocyte culture.
normal thyroid tissue. Clinical studies have also reported, that over 40% of patients treated with tyrosine kinase inhibitors (TKI) developed hypothyroidism [24,25]. Recently Sato et al. reported a sonographically detectable atrophy of the thyroid gland caused by sunitinib therapy [41]. One hypothesis for TKI-related hypothyroidism is inhibition of the VEGF-pathway resulting in reduction of capillary blood flow. These observations and data may signify that VEGF signaling might participate not only in thyroid hyperplasia but is also essential in normal thyroid function as well as physiological proliferation of thyrocytes.

Conclusion ▼

In summary, our study showed an increased expression of VEGF and its receptors in nodular tissue of uninnodular and recurrent goiter as well as in the entire tissue of multinodular goiter and Graves' disease. Therefore, we hypothesized that VEGF may be involved in the proliferation of thyrocytes and its up-regulation via autocrine mechanism might contribute to the development of thyroid nodules and hyperplasia of the thyroid gland. Although, it is very likely that goiter recurrence is of multifactorial origin, VEGF may serve as a proliferation marker in thyroid disease and may be used as a possible target for future strategies for the treatment of neoplastic and autoimmune diseases of the thyroid. Until now, the exact role of VEGF in thyroid function remains to be determined. Hence, more future studies are eminent to elucidate the role of VEGF in thyroid diseases.

Conflict of interest: None.

References

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