

EXPERIMENTAL STUDY

Expression of vascular endothelial growth factor (VEGF) and its receptors in thyroid carcinomas of follicular origin: a potential autocrine loop

Joaquim Miguel Vieira¹, Susana C Rosa Santos¹, Carla Espadinha¹, Isabel Correia¹, Tibor Vag¹, Cristina Casalou¹, Branca Maria Cavaco¹, Ana Luísa Catarino², Sérgio Dias¹ and Valeriano Leite^{1,3}

¹Centro de Investigação de Patobiologia Molecular (CIPM), ²Departamento de Patologia Morfológica, Instituto Português de Oncologia Francisco Gentil – Centro Regional de Oncologia de Lisboa (CROL), S.A, Rua Professor Lima Basto, 1099-023 Lisboa, Portugal and ³Faculdade de Ciências Médicas da Universidade Nova de Lisboa, Lisboa, Portugal

(Correspondence should be addressed to V Leite, CIPM, Instituto Português de Oncologia Francisco Gentil; Email: vleite@ipolisboa.min-saude.pt)

Abstract

Objective: The aim of this study was to clarify the role of vascular endothelial growth factor (VEGF) and VEGF receptor (VEGFR) pathways in thyroid tumourigenesis.

Methods: We examined VEGF, VEGFR-1 and VEGFR-2 expression on 34 papillary thyroid carcinomas (PTCs), 18 follicular thyroid carcinomas (FTCs), eight poorly differentiated thyroid carcinomas (PDTCs) and on a thyroid tumour-derived cell line (NPA'87) by immunohistochemistry, reverse transcriptase PCR, immunofluorescence and Western blotting.

Results: We have demonstrated that VEGF expression was significantly ($P < 0.05$) more prevalent in PTCs (79%) than in FTCs (50%) or PDTCs (37%). Similarly, 76% of PTCs, 83% of FTCs and 25% of PDTCs expressed VEGFR-1, whereas 68% of PTCs, 56% of FTCs and 37% of PDTCs expressed VEGFR-2. Coexpression of VEGF and its receptors was observed in 50% of PTCs, 39% of FTCs and 12% of PDTCs, raising the possibility that VEGF may signal in an autocrine loop in these neoplasias, as observed previously for other types of cancer. In agreement with the idea that autocrine VEGF signalling plays an important role in thyroid carcinogenesis, the blockade of either VEGF or its receptors with neutralizing antibodies significantly reduced cell viability and increased apoptosis levels of the VEGFR-positive thyroid tumour cell line NPA'87.

Conclusions: Our results highlight a previously undefined VEGF autocrine action in thyroid carcinomas which could play a crucial role in tumour cell survival and could represent a useful therapeutic target for thyroid tumours.

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Introduction

Vascular endothelial growth factor (VEGF) plays a key role in tumour angiogenesis, an essential step in tumour growth. VEGF expression is upregulated in the majority of human tumours, where it stimulates proliferation, migration and survival of endothelial cells by binding to two high-affinity tyrosine kinase receptors, VEGFR-1/Flt-1 and VEGFR-2/KDR. VEGF receptors (VEGFRs) are mainly expressed by endothelial cells, although they have also been detected in other cell types, including macrophages, monocytes (1) and platelets (2). More recently, it was shown that both VEGF and its receptors are expressed in haematological (3), pancreatic (4) and prostatic (5) tumour cells, where they may serve as autocrine stimulators of proliferation, migration and survival.

Most thyroid tumours arise from follicular thyroid cells. They comprise a wide spectrum of neoplastic phenotypes, including benign adenomas, well-differentiated papillary and follicular thyroid carcinomas (PTCs and FTCs respectively) and aggressive poorly differentiated and undifferentiated thyroid carcinomas (PDTCs and UTCs respectively). A number of studies have examined the expression of VEGF and its receptors in human thyroid carcinomas with reference to angiogenesis (6–10). VEGF expression is upregulated in thyroid neoplasia, and correlates with the size, spread and recurrence of tumours. For example, VEGF and VEGFR-1 expression correlate with PTC size in children and adults (9), thyroid tumours that have metastasized have higher levels of VEGF than nonmetastatic disease, correlating with a worse prognosis (8), and lymph-node metastases of thyroid carcinomas showed increased VEGF expression compared with primary tumour (7).

Whereas VEGF expression is well documented, much less is known about VEGFR expression in thyroid carcinomas, and some studies obtained contradictory results (6, 8, 9). For instance, some reports describe VEGFR-1 expression as restricted to endothelial cells of normal thyroid tissue, PTCs and FTCs (6, 8). However, others (9) reported VEGFR-1 expression also in PTC cells, raising the possibility of an autocrine mechanism for VEGF on thyroid follicular cells. Expression of VEGFR-2 has been detected in thyroid follicular cells but only at the mRNA level (7).

During the process of malignant transformation, several events have been shown to regulate the expression of VEGF and its receptors, such as hypoxia, p53 deficiency and Ras-activated signal transduction pathway (11). Activating point mutations of *B-Raf*, particularly the Val-600 → Glu (V600E) mutation in the activation segment of the kinase domain, have been found in 29–83% of conventional PTCs but not in FTCs (12–16). To date, the correlation between VEGF, VEGFR expression and *B-Raf* point mutations in thyroid carcinomas has not been analysed.

In this study, we examined the expression pattern and biological function of VEGF and its receptors *in vivo* and *in vitro* to clarify the role of this angiogenic pathway in thyroid tumourigenesis. We demonstrate for the first time the presence of a functional autocrine VEGF/VEGFR pathway that could represent a useful target for therapeutic intervention in thyroid carcinomas.

Materials and methods

Thyroid tissue collection and cell culture

Human thyroid tissues, obtained from fresh surgical samples, were fixed in formalin and embedded in paraffin within 3 h of collection. For the current study, 60 specimens were studied, including 34 PTCs (20 classic variants, 11 follicular variants, 2 tall-cell variants and one cribriform variant), 18 FTCs (15 minimally invasive and three extensively invasive) and eight PDTCs. The extent of PTCs was classified according to the system of DeGroot *et al.* (17) and the metastasis/age/completeness of resection/invasion size score (MACIS; (18)). The system of DeGroot *et al.* (17) categorizes the patients with PTC by clinical class: I, with intrathyroidal disease; II, with cervical adenopathies; III, with extrathyroidal invasion and IV, with distant metastasis. The prognostic score defined as MACIS was calculated as: $MACIS = 3.1$ (if aged ≤ 39 years) or $0.08 \times \text{age}$ (if aged ≥ 40 years), $+ 0.3 \times \text{tumour size (in cm)}$, $+ 1$ (if incompletely resected), $+ 1$ (if locally invasive) and $+ 3$ (if distant metastasis is present).

NPA/87 cells (kindly provided by Dr Danilo V. Canlapan, Los Angeles School of Medicine, Los Angeles, CA, USA) were cultured at 37°C in RPMI 1640 (Life Technologies, Gaithersburg, MD, USA)

supplemented with 10% foetal bovine serum, sodium pyruvate, L-glutamine and penicillin/streptomycin, in a humidified incubator with 5% CO₂.

Immunohistochemical analysis

For immunohistochemistry, 2 µm paraffin-embedded thyroid carcinoma sections were deparaffinized and treated with 0.6% H₂O₂ in methanol to inhibit endogenous peroxidase. Microwave antigen retrieval was used to enhance immunoreactivity. Tissues were blocked with 10% normal goat serum (X907; Dako, Golstrup, Denmark) at room temperature for 10 min, before blocking the endogenous avidin and biotin (SP2001; Vector Laboratories, Burlingame, CA, USA). The antibodies used were polyclonal rabbit anti-human VEGF serum (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal mouse anti-human VEGFR-1 (clone FB5; kindly provided by ImClone Systems, New York, NY, USA) and monoclonal mouse anti-human VEGFR-2 (clone 6.64; kindly provided by ImClone Systems). Primary antibodies were incubated at 4°C overnight. This was followed by sequential incubations with biotin-conjugated secondary antibody, streptavidin/peroxidase and 3,3'-diaminobenzidine (K5001; Dako) for visualization. Sections were counterstained with Mayer's haematoxylin. As negative controls primary antibodies were omitted.

Assessment of microvessel density (MVD)

Blood vessel content in 29 specimens (14 FTCs and 15 PTCs) was determined by immunostaining with a monoclonal rabbit anti-human von Willebrand factor (vWF) antibody (Dako). A modified version of the Weidner method (19) was used for MVD assessment. Briefly, by scanning the tumour sections at low power (40×), the amount of intratumoural vessels was graded from 1 to 4. Then, three areas with the highest vessel density were selected in the tumour, and individual vessels were counted at high power (200×). MVD values represent the average of the assessed 'hotspots'.

Reverse transcriptase (RT)-PCR

Total RNA was reverse-transcribed using Superscript II (Life Technologies). Single-stranded cDNA was amplified using *Taq* polymerase (Life Technologies) as recommended by the manufacturer. *VEGF*, *VEGFR-1* and *VEGFR-2* PCR amplification was done with specific oligonucleotide pairs, described previously (3).

Immunofluorescence analysis

NPA/87 cells were grown on glass coverslips until 70% confluent, washed with PBS, and then fixed in 4% (v/v) formaldehyde/PBS for 5 min at 4°C and washed in PBS. After permeabilization with 0.1% (v/v) Triton X-100

plus 5% (v/v) normal serum, cells were incubated overnight at 4 °C with primary antibodies (polyclonal rabbit anti-human VEGFR-1 or VEGFR-2, both from Santa Cruz Biotechnology). Cells were washed and incubated with secondary antibody (Alexa fluor 594; Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. Samples were mounted in Vectashield (Vector Laboratories) and analysed using fluorescence microscopy (Axioplan Microscope; Zeiss, Cologne, Germany). For negative controls primary or secondary antibodies were omitted.

Protein extraction and Western blotting

Nonidet P-40 lysates were obtained by resuspending the NPA/87 cells in the following buffer: 1% Nonidet P-40, 50 mM Tris, pH 7.5, 10% glycerol and protease/phosphatase inhibitors. Equal protein amounts were separated by SDS/PAGE and transferred to nitrocellulose membranes. Blots were incubated with polyclonal antibodies raised against VEGFR-1, VEGFR-2 or phosphotyrosine (Santa Cruz Biotechnology).

Cell-viability assay

NPA/87 cells were cultured in the presence or absence of the neutralizing monoclonal antibody (mAb) for VEGF (clone 4.6.1 at 100 ng/ml; kindly provided by Genentech, South San Francisco, CA, USA), VEGFR-1 (clone 6.12 at 1 µg/ml; kindly provided by ImClone Systems) or VEGFR-2 (clone IMC1C11 at 1 µg/ml; kindly provided by ImClone Systems). After 24, 48 and 72 h, viable and dead cells were counted in triplicate, based on trypan blue exclusion. Each experimental condition was performed in duplicate, and experiments were repeated three times.

Apoptosis studies

NPA/87 cells were treated with vehicle or neutralizing mAb to VEGF (clone 4.6.1), VEGFR-1 (clone 6.12) or VEGFR-2 (clone IMC1C11) for 24 h. Cells were

washed once in the incubation buffer (10 mM HEPES, pH 7.4, 10 mM NaCl and 5 mM CaCl₂) and resuspended in incubation buffer supplemented with 0.5 mg/ml propidium iodide (Sigma-Aldrich, St Louis, MO, USA) and annexin V-FITC (Boehringer Mannheim, Mannheim, Germany) at room temperature for 30 min. The percentage of early apoptotic cells (annexin V-FITC-positive, propidium iodide-negative) was determined by flow cytometry (FACScan; Becton-Dickinson, San Jose, CA, USA). These experiments were repeated three times.

Cell-cycle analysis

NPA/87 cells were treated as described for apoptosis studies, washed in cold PBS containing 5 mM EDTA, then incubated in the above buffer supplemented with 0.2 mg/ml RNase A, at room temperature, for 30 min. Propidium iodide solution (5 µg/ml) was added to the samples and cell cycle was analysed by flow cytometry. DNA histograms were obtained by using Multicycle software (Phoenix Flow Systems, San Diego, CA, USA).

Mutation screening of B-Raf

We performed mutation analysis in exon 15 of *B-Raf* in 20 conventional PTCs. The complete coding sequences of exon 15 of *B-Raf* were amplified using exonic primers and directly sequenced on both strands using the ABI PRISM[®] Big Dye[™] Terminator Cycle Sequencing Ready Reaction Kit and analysed by the ABI PRISM[™] 310 Genetic Analyser (Applied Biosystems, Foster City, CA, USA). The primer (5'–3') sequences were as follows: *B-Raf* forward primer, GCC AAG TCA ATC ATC CAC AG; *B-Raf* reverse primer, CAT CTG ACT GAA AGC TGT ATG GA.

Statistical analysis

All graphs with error bars indicate mean ± S.E.M. The relationship between VEGF immunoreactivity and MVD was assessed using the Spearman correlation test. VEGF and VEGFR immunostaining was compared

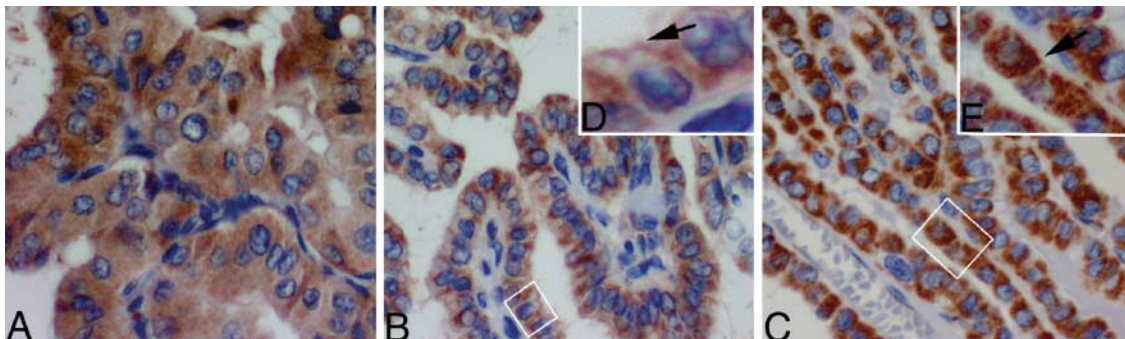


Figure 1 Immunohistochemical detection of VEGF (A), VEGFR-1 (B, D) and VEGFR-2 (C, E) in cytoplasm and on the surface (D, E) of thyroid carcinoma cells. Arrows indicate VEGFRs staining in cell membrane. Panels are a series from three different thyroid carcinomas. Original magnifications, A–C, × 400; D and E, × 600.

between different tumour histotypes by two-tailed Fisher's exact test. The correlation of VEGF and VEGFR expression with clinical/pathological features of the patients or the presence of *B-Raf* point mutations was performed using unpaired *t*-test, two-tailed Fisher's exact test or χ^2 test, as appropriate. Differences between experimental and control groups in cell viability, apoptosis and cycle assays were evaluated by one-way analysis of variance (ANOVA), followed by Tukey's *post-hoc* test. Statistical analysis was performed using GraphPad Prism statistical software (San Diego, CA, USA). Differences were considered statistically different at $P < 0.05$.

Results

Expression of VEGF and VEGFRs in human thyroid carcinomas

VEGF immunohistochemical staining was detected in 79% (27/34) of PTCs ($P = 0.03$ versus FTC; $P = 0.02$ versus PDTC), 50% (9/18) of FTCs ($P > 0.05$ versus PDTC) and 37% (3/8) of PDTCs. The VEGF immunoreactivity was always confined to the cytoplasm of epithelial cells (Fig. 1), but an occasional inflammatory

cell staining was also observed (data not shown). VEGF levels in FTCs and PTCs were compared with MVD. The correlation between VEGF expression and MVD was statistically significant in PTCs ($r = 0.747$; $P = 0.0014$) but not in FTCs ($r = 0.333$; $P > 0.05$; Fig. 2).

Immunohistochemical staining with antibodies specific for VEGFR-1 or VEGFR-2 revealed that, besides staining the endothelial lining of blood vessels, subsets of carcinoma cells also expressed the receptors (Fig. 1). VEGFR-1 immunoreactivity was detected in 76% (26/34) of PTCs ($P > 0.05$ versus FTC; $P = 0.006$ versus PDTC), 83% (15/18) of FTCs ($P = 0.004$ versus PDTC) and 25% (2/8) of PDTCs, whereas VEGFR-2 was detected in 68% (23/34) of PTCs ($P > 0.05$ versus FTC or PDTC), 56% (10/18) of FTCs ($P > 0.05$ versus PDTC) and 37% (3/8) of PDTCs. In general, diffuse cytoplasmatic and membrane VEGFRs immunoreactivity was observed in thyroid tumour cells (Fig. 1). Coexpression of VEGF and its receptors was observed in 50% (17/34) of PTCs ($P > 0.05$ versus FTC or PDTC), 39% (7/18) of FTCs ($P > 0.05$ versus PDTC) and 12% (1/8) of PDTCs.

Relationship between clinicopathological data and the expression of VEGF and its receptors in human thyroid carcinomas

FTCs expressing VEGF, VEGFR-1, VEGFR-2 or both VEGF and its receptors (VEGF + VEGFRs) were not significantly larger or more invasive than those lacking its expression (Table 1). In PTC cases (Table 2), we observed that 95% (19/20) of the tumours with a classic pattern were positive for VEGFR-1, whereas only 45% (5/11) of the follicular variants were positive ($P = 0.007$). Additionally, we observed that 65% (13/20) of conventional PTCs were positive for both VEGF and its receptors, whereas only 18% (2/11) of the follicular variants expressed both VEGF and VEGFRs ($P = 0.03$). Interestingly, 100% (5/5) of the tumours positive for both VEGF and VEGFRs had metastasized to neck lymph nodes (class II tumours), whereas only 32% (6/19) of intrathyroidal tumours (class I tumours) were positive ($P = 0.04$).

Mutation screening of B-Raf

Since VEGF and its receptors were mainly expressed by conventional PTCs, we next investigated whether there was a correlation with the presence of *B-Raf* mutations, a common event in this kind of tumours. We detected the *B-Raf*^{V600E} mutation in 50% (10/20) of conventional PTCs, but there was no significant correlation between the presence of the *B-Raf*^{V600E} mutation and the expression of VEGF and its receptors (Table 3).

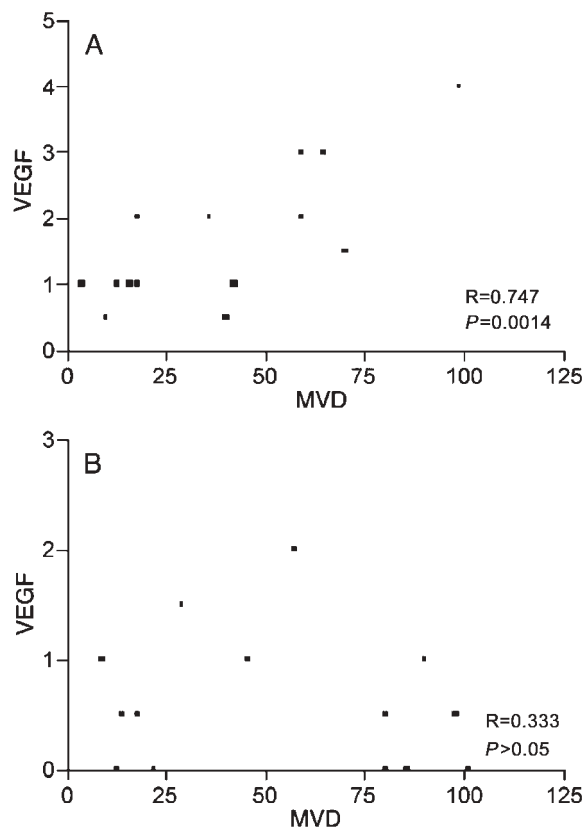


Figure 2 Correlation between MVD and VEGF expression in PTCs (A) and FTCs (B). Spearman correlation test was used to determine r and P values.

Table 1 Clinical and pathological features and VEGF/VEGFR expression in FTCs.

| | VEGF | | | VEGFR-1 | | | VEGFR-2 | | | VEGF + VEGFRs | | |
|------------------------------|----------------|----------|---------|----------------|----------|---------|----------------|----------|---------|----------------|----------|---------|
| | Immunostaining | | P value | Immunostaining | | P value | Immunostaining | | P value | Immunostaining | | P value |
| | Negative | Positive | | Negative | Positive | | Negative | Positive | | Negative | Positive | |
| Tumour size (cm±s.d.) | 3.4±1.9 | 4.9±1.3 | NS | 4.3±1.3 | 3.9±1.9 | NS | 3.4±1.1 | 4.8±2.0 | NS | 3.6±1.8 | 5.1±1.1 | NS |
| Invasiveness | | | NS | | | NS | | | NS | | | NS |
| Minimally invasive (n = 15) | 8 (53%) | 7 (47%) | | 3 (20%) | 12 (80%) | | 8 (53%) | 7 (47%) | | 10 (67%) | 5 (33%) | |
| Extensively invasive (n = 3) | 1 (33%) | 2 (67%) | | 0 (0%) | 3 (100%) | | 0 (0%) | 3 (100%) | | 1 (33%) | 2 (67%) | |
| Distant metastasis (n = 3) | 1 (33%) | 2 (67%) | NS | 0 (0%) | 3 (100%) | NS | 0 (0%) | 3 (100%) | NS | 1 (33%) | 2 (67%) | NS |
| Clinical status | | | | | | | | | | | | |
| Persistent (n = 3) | 1 (33%) | 2 (67%) | NS | 0 (0%) | 3 (100%) | NS | 0 (0%) | 3 (100%) | NS | 1 (33%) | 2 (67%) | NS |
| Remission (n = 15) | 8 (53%) | 7 (47%) | | 3 (20%) | 12 (80%) | | 8 (53%) | 7 (47%) | | 10 (67%) | 5 (33%) | |

NS, not significant.

Table 2 Clinical and pathological features and VEGF and VEGFR expression in PTCs.

| | VEGF | | | VEGFR-1 | | | VEGFR-2 | | | VEGF + VEGFRs | | |
|------------------------------|----------------|----------|---------|----------------|----------|---------|----------------|----------|---------|----------------|----------|---------|
| | Immunostaining | | P value | Immunostaining | | P value | Immunostaining | | P value | Immunostaining | | P value |
| | Negative | Positive | | Negative | Positive | | Negative | Positive | | Negative | Positive | |
| Tumour size (cm±s.d.) | 3.7±1.8 | 3.8±2.7 | NS | 3.5±1.4 | 3.9±2.8 | NS | 3.8±1.4 | 3.8±2.9 | NS | 4.1±1.6 | 3.7±1.8 | NS |
| MACIS (mean±s.d.) | 5.6±1.8 | 5.4±1.6 | NS | 4.9±0.9 | 5.6±1.6 | NS | 5.2±1.0 | 5.7±1.9 | NS | 5.6±1.7 | 5.8±1.9 | NS |
| Class | | | NS | | | NS | | | NS | | | 0.04 |
| I (n = 19) | 5 (26%) | 14 (74%) | | 7 (37%) | 12 (63%) | | 8 (42%) | 11 (58%) | | 13 (68%) | 6 (32%) | |
| II (n = 5) | 0 (0%) | 5 (100%) | | 0 (0%) | 5 (100%) | | 0 (0%) | 5 (100%) | | 0 (0%) | 5 (100%) | |
| III (n = 8) | 1 (12%) | 7 (88%) | | 1 (12%) | 7 (88%) | | 3 (37%) | 5 (63%) | | 3 (37%) | 5 (63%) | |
| IV (n = 2) | 1 (50%) | 1 (50%) | | 0 (0%) | 2 (100%) | | 0 (0%) | 2 (100%) | | 1 (50%) | 1 (50%) | |
| Predominant pattern | | | NS | | | 0.007 | | | NS | | | 0.03 |
| Classic (n = 20) | 3 (15%) | 17 (85%) | | 1 (5%) | 19 (95%) | | 5 (25%) | 15 (75%) | | 7 (35%) | 13 (65%) | |
| Follicular (n = 11) | 3 (27%) | 8 (73%) | | 6 (55%) | 5 (45%) | | 5 (45%) | 6 (55%) | | 9 (82%) | 2 (18%) | |
| Others (n = 3) | 1 (33%) | 2 (67%) | | 1 (33%) | 2 (67%) | | 1 (33%) | 2 (67%) | | 1 (33%) | 2 (67%) | |
| Clinical status ^a | | | NS | | | NS | | | NS | | | NS |
| Persistent (n = 6) | 1 (17%) | 5 (83%) | | 1 (17%) | 5 (83%) | | 2 (33%) | 4 (67%) | | 2 (33%) | 4 (67%) | |
| Remission (n = 27) | 6 (22%) | 21 (78%) | | 7 (26%) | 20 (74%) | | 9 (33%) | 18 (67%) | | 15 (56%) | 12 (44%) | |

NS, not significant.

^a Loss of follow-up of one patient.

Table 3 Correlation of *B-Raf* mutations with VEGF and VEGFR expression in conventional PTCs.

| | VEGF | | | VEGFR-1 | | | VEGFR-2 | | | VEGF + VEGFRs | | |
|-------------------------------|----------------|----------|----------------|----------------|-----------|----------------|----------------|----------|----------------|----------------|----------|----------------|
| | Immunostaining | | | Immunostaining | | | Immunostaining | | | Immunostaining | | |
| | Negative | Positive | <i>P</i> value | Negative | Positive | <i>P</i> value | Negative | Positive | <i>P</i> value | Negative | Positive | <i>P</i> value |
| <i>B-Raf</i> ^{V600E} | | | NS | | | NS | | | NS | | | NS |
| Positive (<i>n</i> = 10) | 2 (20%) | 8 (80%) | | 0 (0%) | 10 (100%) | | 3 (30%) | 7 (70%) | | 3 (30%) | 7 (70%) | |
| Negative (<i>n</i> = 10) | 1 (10%) | 9 (90%) | | 1 (10%) | 9 (90%) | | 2 (20%) | 8 (80%) | | 4 (40%) | 6 (60%) | |

NS, not significant.

A VEGF/VEGFR autocrine loop in NPA'87 cells is crucial for survival

The coexpression of VEGF and VEGFRs on thyroid carcinomas of follicular origin, particularly in conventional PTCs, suggests the presence of a VEGF autocrine loop. In order to test this hypothesis, we used specific neutralizing antibodies to block the VEGF/VEGFR pathway in a human papillary thyroid carcinoma-derived cell line, NPA'87. These cells express the three VEGF isoforms (VEGF-121, VEGF-165 and VEGF-189) as well as both VEGFRs; VEGFR-1/Flt-1 and VEGFR-2/KDR (Fig. 3A). The VEGFR expression was further characterized by immunofluorescence (Fig. 3B). VEGFR-1 staining was evident both in the cytoplasm and on the cell membrane of NPA'87 cells, while VEGFR-2 staining, also evident in the cytoplasm, showed a more prominent perinuclear pattern (Fig. 3B). However, only VEGFR-2 seems to be functionally active on NPA'87 cells, as demonstrated by Western blotting (Fig. 3C). After stimulation with VEGF, no VEGFR-1 phosphorylation was observed (Fig. 3C). Noteworthy, in the absence of exogenous VEGF, baseline VEGFR-2 phosphorylation was observed in NPA'87 cells (Fig. 3C), further suggesting the existence of a functional autocrine loop in these cells.

Next, we investigated the potential role of the VEGF/VEGFR autocrine interaction in cell survival and cycle progression. NPA'87 cells were grown in serum-free conditions and treated with neutralizing antibodies specific for VEGF or its receptors. We observed that blocking either VEGF (VEGF mAb; 100 ng/ml) or VEGFR-2 (VEGFR-2 mAb; 1 µg/ml) significantly ($P < 0.05$) reduced cell viability (live/dead ratio) by 60% (Fig. 4A), when compared with control cells (treated with vehicle), after 24 h of treatment. The blockade of VEGFR-1 (VEGFR-1 mAb; 1 µg/ml) also decreased cell viability by 40% compared with untreated cells ($P < 0.05$). Subsequently, we evaluated cell death and cell-cycle progression in NPA'87 cells treated with blocking antibodies. Using annexin V-FITC- and propidium iodide-binding assay, we recorded a 2.5-fold increase in the apoptotic cell population (annexin V-FITC-positive, propidium iodide-negative) after treatment with VEGF-, VEGFR-1- or VEGFR-2-neutralizing antibodies ($P < 0.05$; Fig. 4B), compared

with untreated cells. Conversely, cell-cycle analysis revealed a modest arrest of cells in $G_0 + G_1$ phase, increasing approximately 17–21% compared with controls cells, and a decrease of cells in G_2 phase by 26–40% (Fig. 4C), independent of the treatment used. These results suggest that the VEGF/VEGFR system plays a crucial role in thyroid tumour cell survival.

Discussion

VEGF, an important stimulator of angiogenesis, has been implicated in proliferation and spread of different malignant tumours. In thyroid carcinomas of follicular origin, the role of VEGF is not fully understood. Yet, VEGF overexpression in thyroid tumours appears to be related to some clinical/pathological data of the patients (6–10). VEGF overexpression correlated with PTC size in adults and children (9), and higher VEGF expression correlated with tumour metastatic behaviour and recurrence (8). In the present report, VEGF staining was found to be significantly more frequent in PTCs than in FTCs or PDTs. Our results are in concordance with other reports (10), which also showed downregulation of VEGF in PDTs. This finding suggests that VEGF plays an important role in the early stages of thyroid tumourigenesis, where it is overexpressed, but during progression to more undifferentiated stages, like PDTs, other growth factors may be more important. The present study also revealed a highly significant relationship between the expression of VEGF and blood-vessel content in PTCs. This finding was contrary to MVD in FTCs, where we could not show a correlation with VEGF levels, and it is in agreement with a recent report (20). These results indicate that VEGF sustains tumour angiogenesis in PTCs, where in FTCs other angiogenic factors may be involved.

VEGF signals via two high-affinity tyrosine kinase receptors, VEGFR-1/Flt-1 and VEGFR-2/KDR, expressed mostly in endothelial cells. Recently, VEGFR expression has been reported in various types of tumour, such as leukaemia (3), pancreatic carcinoma (4) and prostatic carcinoma (5), suggesting a potential autocrine/paracrine VEGF action in these tumours. In thyroid carcinomas, VEGFR expression has been reported mainly in

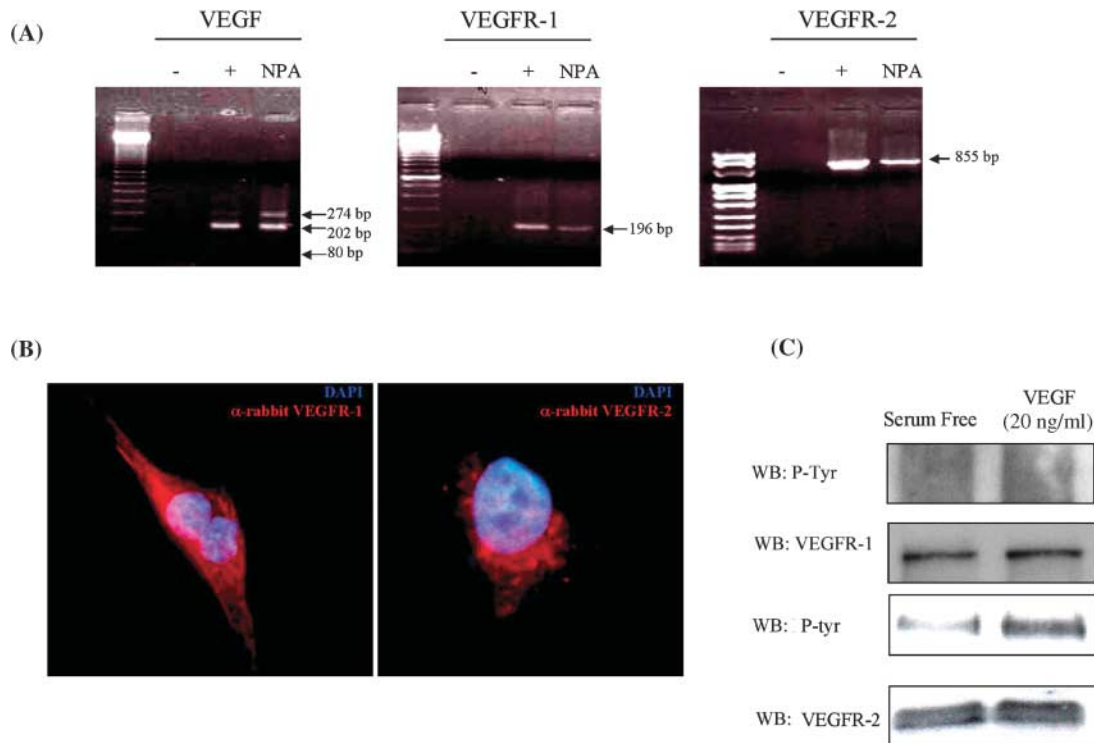


Figure 3 VEGF, VEGFR-1 and VEGFR-2 expression by NPA/87 cells. (A) RT-PCR analysis for VEGF, VEGFR-1 and VEGFR-2 in NPA/87. As a negative control (–), PCR was performed without template, whereas HL60 cDNA (VEGF) and human umbilical-vein endothelial cell cDNA (VEGFRs) were used as positive controls (+). For VEGF, the indicated bands 80, 202 and 274 bp correspond to VEGF-121, VEGF-165 and VEGF-189, respectively. For VEGFRs, the 196 bp band corresponds to VEGFR-1, whereas the 855 bp band corresponds to VEGFR-2. (B) Immunofluorescence analysis of NPA/87 cells in serum-free medium. These cells were stained for VEGFR-1 and VEGFR-2 (phycoerythrin) and DNA (nucleus; DAPI, 4',6-diamidino-2-phenylindole). Note the membrane and cytoplasmic (prominent perinuclear pattern for VEGFR-2) expression of VEGFRs. This result was seen in most (95%) cells in each experiment, and was repeated three times. Negative controls included primary antibody alone and secondary antibody alone (data not shown; original magnification, $\times 630$). (C) Western blot (WB) analysis of total protein extracts from NPA/87 cells in the presence or absence (serum-free conditions) of VEGF, stained against VEGFR-1, VEGFR-2 and phosphotyrosine (p-Tyr). Note the increased phosphorylation of VEGFR-2 after VEGF stimulation (20 ng/ml) for 10 min.

endothelial cells (6, 8). However, one study (9) indicated that, besides endothelial cells, VEGFR-1 might also be expressed by PTC cells. In the present study we confirm that both VEGFRs are expressed by thyroid epithelium-derived tumour cells, in addition to the endothelial cells of tumour blood vessels. This is the first demonstration of VEGFR-2 protein expression in thyroid carcinomas. Moreover, we show that coexpression of VEGF, VEGFR-1 and VEGFR-2 was significantly more prevalent in classical variants than in follicular variants of PTCs, being associated with lymph-node dissemination.

We hypothesized that the presence of specific VEGFRs on thyroid carcinoma cells would allow such cells to respond to VEGF in either an autocrine or paracrine manner. To confirm this, we used a thyroid tumour-derived cell line, NPA/87, which expresses both VEGF and VEGFRs, as confirmed by RT-PCR, immunofluorescence and Western blotting. We investigated whether cellular processes, such as survival and cell-cycle progression were affected by inhibition of the VEGF pathway. Blockade of VEGF or VEGFRs by neutralizing

mAbs resulted in a decreased cell viability, which was more pronounced with VEGFR-2 mAb than with VEGFR-1 mAb. This suggests that the biological action of VEGF is mainly mediated through VEGFR-2. Blockade of VEGF/VEGFR system also induced apoptosis, but had a minor effect on cell-cycle progression. Hence, this study demonstrates a functional VEGF autocrine loop that contributes to tumour-cell survival, and suggests that VEGF- and VEGFR-specific inhibitors could be a promising approach that should be explored for treatment of thyroid tumours. Several reports have been published in support of this idea (21–23). For instance, Vatalanib (PTK787/ZK222584), a specific blocker of VEGFRs (tyrosine kinase activity), was shown to inhibit the growth of thyroid carcinomas in an animal study by blocking angiogenesis (21), whereas in colorectal cancer, treatment with Bevacizumab, a VEGF-neutralizing antibody, suggested a real clinical importance (22). ZD6474, an inhibitor of VEGFR-2/KDR tyrosine kinase activity, has also been shown to efficiently block oncogenic RET kinases, arresting

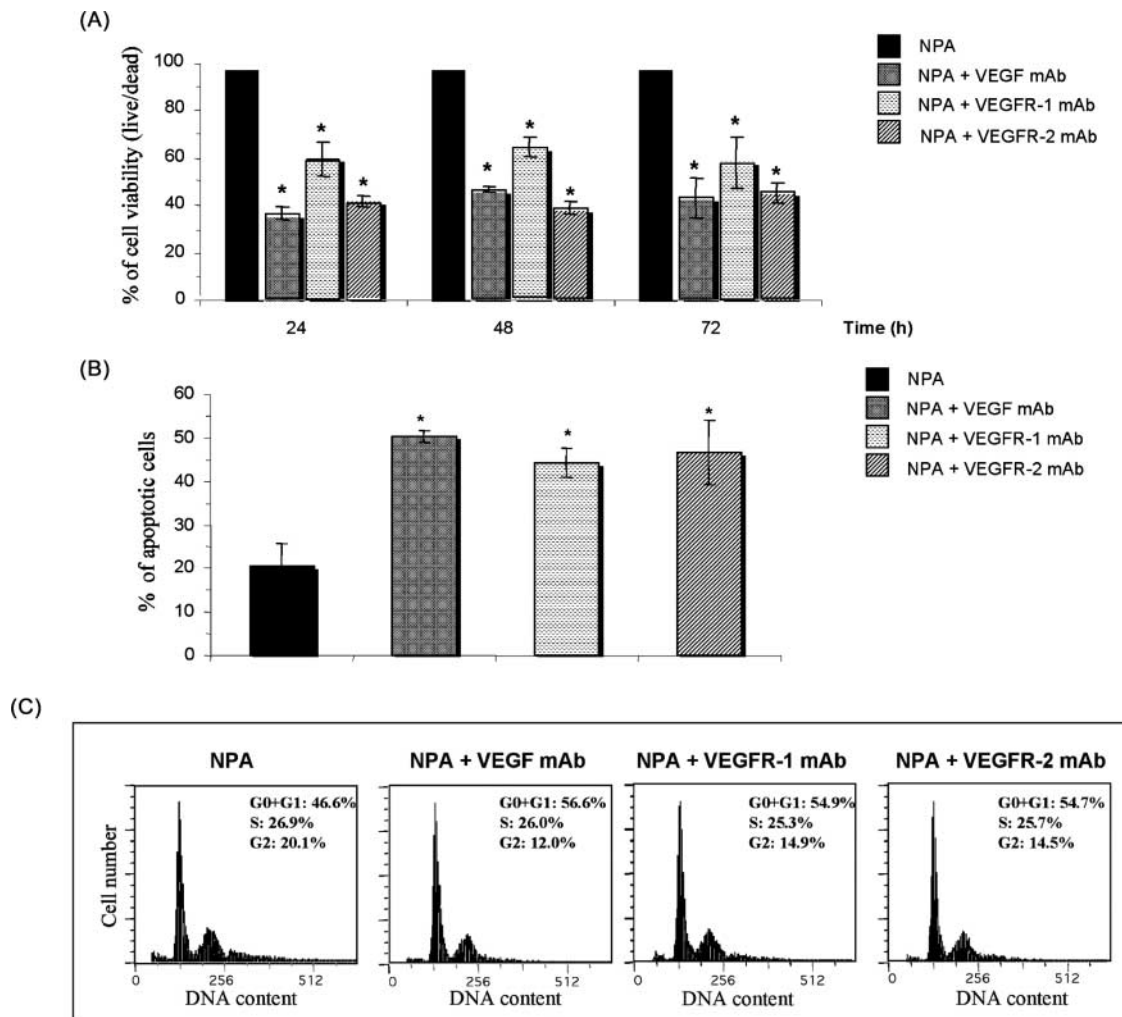


Figure 4 Blocking the VEGF/VEGFR autocrine loop exerts an effect on NPA/87 cell viability, apoptosis and cell-cycle progression. Graphs with error bars indicate means \pm S.E.M. (A) NPA/87 cells were left untreated (in serum-free conditions) or treated with VEGF, VEGFR-1 or VEGFR-2 mAbs for 24, 48 and 72 h. Live and dead cells (determined by Trypan Blue staining) were counted using a haemocytometer. Results show the ratio of live/dead cells in the different conditions, normalized against the untreated group. Treatment of NPA/87 cells with VEGF, VEGFR-1 or VEGFR-2 mAbs decreases cell viability ($*P < 0.05$, at 24, 48 and 72 h), with VEGF and VEGFR-2 mAbs having a more significant effect than VEGFR-1 mAb ($*P < 0.05$, at 24 and 48 h). The results shown are representative of three separate experiments. (B) Untreated (control) or treated (24 h) NPA/87 cells with neutralizing mAbs against VEGF and its receptors, were stained with annexin V-FITC and propidium iodide, and analysed by flow cytometry (FACScan). Results show the percentage of early apoptotic cells (annexin V-FITC-positive, propidium iodide-negative) in the different conditions. As shown, blockade of either VEGF or its receptors induces apoptosis in NPA/87 cells ($*P < 0.05$). These results are representative of three independent experiments. (C) NPA/87 cells treated as before were stained with propidium iodide and analysed by flow cytometry. Results show the percentage of cells in each cell-cycle phase (G₀ + G₁, S and G₂ phases).

RET/PTC3-induced tumour growth in nude mice, in addition to tumoural angiogenesis blockade (23).

Finally, we speculated about the mechanisms underlying the expression of VEGF and VEGFRs in thyroid carcinoma cells. Several events have been shown to regulate the expression of VEGF, such as hypoxia, p53 deficiency and Ras-activated signal transduction pathway (11). In fact, stimulation of the classical Ras/Raf/mitogenactivated protein kinase/extracellular-signalregulated kinase (ERK) kinase (MEK)/ERK1/2 pathway, by expression of oncogenic Ras, inducible Raf

or constitutively active MEK, results in an enhanced VEGF promoter activity (24). *B-Raf* mutations, particularly the V600E mutation, are specifically present in classical PTCs (12–16). Taking these data into consideration, we hypothesized that mutations in *B-Raf* could be correlated with VEGF and VEGFR expression in classical PTCs. We performed mutational analysis and detected *B-Raf*^{V600E} mutations in 50% of classical PTC, a percentage that is in agreement with previous reports (12–16). However, we did not find a correlation between the presence of *B-Raf*^{V600E} mutation and the

expression of VEGF or VEGFRs in PTCs. These results suggest that other mechanisms are responsible for VEGF and VEGFR overexpression in thyroid carcinomas.

In summary, coexpression of VEGF and its receptors in thyroid carcinomas, particularly in conventional PTCs, demonstrates the existence of a VEGF autocrine signalling pathway, which could contribute to tumour cell survival. Given its potential at blocking cell viability and the existence of clinically approved agents to block it, the VEGF/VEGFR pathway could represent a useful target for therapeutic intervention in thyroid carcinomas.

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