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ORIGINAL ARTICLE

Interrelated overexpression of endothelial and inducible nitric oxide synthases, endothelin-1 and angiogenic factors in human papillary thyroid carcinoma

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Summary

Objective Nitric oxide (NO) and endothelin-1 (ET-1) are involved in carcinogenesis. Overexpression of the ET-1 axis has been demonstrated in papillary thyroid carcinoma (PTC). This study investigated the expression of NO synthases (NOS) and their relationship with expression of ET-1 and angiogenic markers in PTC.

Design and Patients Expression of NOS, angiogenic markers [vascular endothelial growth factor (VEGF), angiopoietin-1 and angiopoietin-2] and their receptors was studied in surgical thyroid samples obtained from 22 patients aged 15–68 years. Three groups were constituted: normal thyroid ($n = 5$), Hashimoto's thyroiditis ($n = 9$) and PTC ($n = 8$).

Results Immunohistochemistry disclosed NOS2 and NOS3 immunoreactivity in PTC cells, the percentage of positive cells being greater than normal ($P < 0{\cdot}02$). Real-time quantitative polymerase chain reaction (RTQ-PCR) showed that NOS2 and NOS3 mRNA levels were, respectively, increased ($P < 0{\cdot}02$) by $2{\cdot}6 \pm 0{\cdot}6$ and $4{\cdot}2 \pm 1{\cdot}1$ times in PTC. RTQ-PCR demonstrated that VEGF, its receptors VEGFR-1 and VEGFR-2, and angiopoietin-2 and its receptor (Tie2) were also overexpressed ($P < 0{\cdot}05$) in PTC. Correlations were found between ET-1 expression and that of NOS2, angiopoietin-1 and -2 ($P < 0{\cdot}05$). NOS2 mRNA levels also correlated with those of NOS3 and angiopoietin-2 ($P < 0{\cdot}05$). In thyroiditis, NOS2 immunoreactivity was observed in inflammatory cells whereas NOS2 mRNA levels were $12{\cdot}1 \pm 1{\cdot}6$ times higher than normal ($P < 0{\cdot}005$).

Conclusions This study revealed an activation of the NO pathway in thyroid carcinoma, which is interrelated to the ET-1 axis, both systems being overexpressed in concert with angiogenic factors. This global system might play a role in carcinogenesis and constitutes a potential target for anticancer therapy.

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Introduction

Endothelin-1 (ET-1) and nitric oxide (NO) are now recognized as important endothelial-derived vasomediators, the former inducing vasoconstriction, the latter vasodilation.[1,2] In addition to their role in modulating vascular tone, both could be involved in various processes affecting carcinogenesis.

Papillary and medullary thyroid carcinomas are cancers in which we recently demonstrated overexpression of the ET-1 axis, including the ET-1 precursor, its mitogenic receptor ET_A and ET-1 converting enzyme.[3–5] ET-1 could play a deleterious role in these cancers by promoting tumour cell survival and proliferation as well as angiogenesis and bone metastases.[6]

NO is a free radical molecule with various functions, including vasodilation and also neurotransmission and immune regulation.[7] NO is synthesized by the NO synthases (NOS), which comprise three isoforms, two of which are constitutive [the endothelial NOS (NOS3) and the neuronal NOS (NOS1)] while the third is inducible (NOS2). Several studies have shown that the three isoforms can be detected in cancer cells from different isolates and might be involved in promoting or inhibiting the pathophysiology of cancer.[7–12] There have been very few significant studies evaluating NOS expression in thyroid carcinomas.[13,14] These studies only reported immunohistochemical staining for NOS in thyroid cancer but without quantifying their expression at a molecular level.

Furthermore, angiogenesis is an important process in tumour growth and metastasis, where ET-1 and NO could play a role. Indeed, ET-1 has been shown to stimulate endothelial cell growth and to induce vascular smooth muscle cell and pericyte mitogenesis.[15] ET-1 can also induce vascular endothelial growth factor (VEGF) by increasing hypoxia-inducible factor-1α, as was demonstrated in ovarian carcinoma cells.[16] In breast cancer, ET-1 expression has been correlated with neovascularization and with the expression of VEGF.[17] NO is also able to regulate VEGF and angiogenesis, as

demonstrated by the increase in vessel density and tumour growth in NOS2-transfected tumours.[18] Besides VEGF, angiopoietins are other important molecules that are implicated in the control of angiogenesis.[19,20] However, VEGF and angiopoietins have never been studied in connection with ET-1 and NO in thyroid cancer.

The first aim of the present study was to investigate and quantify the expression of the three NOS isoforms together in a series of papillary thyroid carcinoma (PTC) in comparison with Hashimoto's thyroid and normal thyroid. Because of the physiological reciprocal interactions between NO and ET-1, the second aim of this study was to determine whether the activation of one of these two systems is balanced by the other one in thyroid cancer. The third aim was to examine how the expression of angiogenic markers, such as VEGF, angiopoietins and their receptors, was activated in relation to that of ET-1 and/or NO. Both real-time quantitative polymerase chain reaction (RTQ-PCR) and immunohistochemical studies were used for this purpose.

Methods

Patients and protocol

Surgical human thyroid samples were obtained from 22 patients (20 women and two men) in whom the ET-1 axis had been studied previously.[3–5] The patients' ages ranged from 15 to 68 years (mean 42·9 ± 3·1 years). Surgery was carried out by a single surgeon under the same conditions and using the same surgical technique. Thyroid samples were divided into three groups according to histological examination: normal thyroid ($n = 5$; age of patients: 44·6 ± 4·0 years), Hashimoto's thyroiditis ($n = 9$; age: 45·8 ± 5·8 years) and PTC ($n = 8$; age: 38·7 ± 5·0 years). Normal thyroid samples were obtained from normal thyroid tissue at a distance from a benign nodule. Tissue samples were divided into three pieces: one was fixed in formalin fluid for conventional histology and immunohistochemistry; the others were fixed in liquid nitrogen immediately after surgical excision, and were then stored at −80 °C and processed later for RTQ-PCR.

Immunohistochemistry, scores for immunostaining and morphometry

Immunohistochemistry was performed for histological detection of NOS2 and NOS3. The immunolocalizations were realized on routine 5-μm-thick sections of paraffin-embedded formalin-fixed thyroid specimens. After deparaffinization and rehydration, sections were reacted for 15 min with 3% hydrogen peroxide in 100% methanol to inhibit endogenous peroxidase activity. The sections were then rehydrated and those for NOS2 detection were submitted to antigen retrieval treatment. This consisted of putting sections in 0·01 M citrate buffer (pH 6·0) into a microwave oven (one cycle of 11 min, at 650 W). The sections were left at room temperature for 20 min and then rinsed in phosphate-buffered saline (PBS; pH 7·4, 10 min). The sections were then incubated for 1 h at room temperature with either anti-NOS2 antibodies (polyclonal rabbit anti-NOS2, RB-9242-P, Laboratory Vision Corporation, dilution 5 μg/ml) or anti-NOS3 antibodies [monoclonal mouse immunoglobulin G1 (IgG1) anti-NOS3, 610297, BD Transduction Laboratories, dilution 2·5 μg/ml] diluted in PBS supplemented with 1% bovine serum albumin (BSA). It is noteworthy that none of the peptides used to immunize the animals was coupled to thyroglobulin (which is classically used to enhance the immune response). After three successive washings with PBS supplemented with 0·1% BSA, the sections were incubated for 30 min with Envision Rabbit System (K4002, Dako, Carpenteria, CA, USA) or with Envision Mouse System (K4000, Dako) for NOS2 and NOS3, respectively. After washings as described above, the peroxidase activity was determined by using a commercial aminoethylcarbazole substrate kit (AEC+, K3461, Dako), giving a red signal, and the sections were counterstained with Mayer's haematoxylin (blue colour), and finally mounted with an aqueous mounting medium (Faramount, S3025, Dako).

The results of the immunohistochemistry were evaluated by counting the number of positive follicular cells among 1000 follicular cells. The intensity of the immunostaining of the follicular cells was also graded semiquantitatively; the grades were 0 for no staining, 1 for weak staining and 2 for strong staining. These grades were then multiplied by the number of positive cells per 100 cells in each category (weak or strong staining) and each group to provide an immunohistochemical score. The two categories were summed to obtain a global score in each group that was used for statistical analysis.

As described previously,[3] a morphometric evaluation was performed to determine the volumic fraction of four thyroid compartments (follicular cells, follicular lumens, cells from inflammatory infiltrates, and other structures including vessels, connective tissue, etc.).

RTQ-PCR

RTQ-PCR was used to quantify transcripts of NOS1, NOS2, NOS3, VEGF, VEGF receptors (VEGFR-1 or Flt-1, VEGFR-2 or Flk-1/KDR), angiopoietin-1, angiopoietin-2 and angiopoietin receptor (Tie2). Their expression was normalized using the hypoxanthine phosphoribotransferase (HPRT) housekeeping gene product as an endogenous reference. RNA isolation, reverse transcription and RTQ-PCR were performed as described previously.[3] The results were expressed using the ΔΔCt methods after validation, following the recommendations of the manufacturer (Perkin-Elmer Applied Biosystems). The primers, obtained from GenBank, were as follows: NOS3, forward 5′-TTGGCGGCGGAAGAGGAAGGAGT-3′, reverse 5′-CAAAGGCGCAGAAGTGGGGGTATG-3′ (slope −3·17, $r = 0·99$, NM_000603); NOS2, forward 5′-ACGTGCGTTACTCAACAAACAA-3′, reverse 5′-CATAGCGGATGAGCTAGCATT-3′ (slope −3·07, $r = 0·98$, NM_153292); NOS1, forward 5′-CCTCCCGCCCTGCACCATCTT-3′, reverse 5′-CTTGCCCCATTTCCATTCCTCGTA-3′ (slope −3·11, $r = 0·99$, NM_000620); VEGF, forward 5′-TACCTCCACCATGCAAGTGG-3′, reverse 5′-GTAGCTGCGCTGGTAGACGTC-3′ (slope −3·08, $r = 0·94$, M32977); VEGFR-1, forward 5′-AACTAGGCGACCTGCTGCAA-3′, reverse 5′-AGTGGAGTACGTGAAGCCGC3′ (slope −2·92, $r = 0·98$, AJ245445); VEGFR-2, forward 5′-ACTGGTTCTGGCCCAACAATC-3′, reverse 5′-CAGAAGAAGCCGTCACTGCA-3′ (slope −3·49, $r = 0·94$, AJ245446); angiopoietin-1, forward 5′-GGCAACTGTCGTGAGAGTACGA-3′, reverse 5′-CATTTAGATTGGAGGGGCCACA-3′ (slope −2·88,

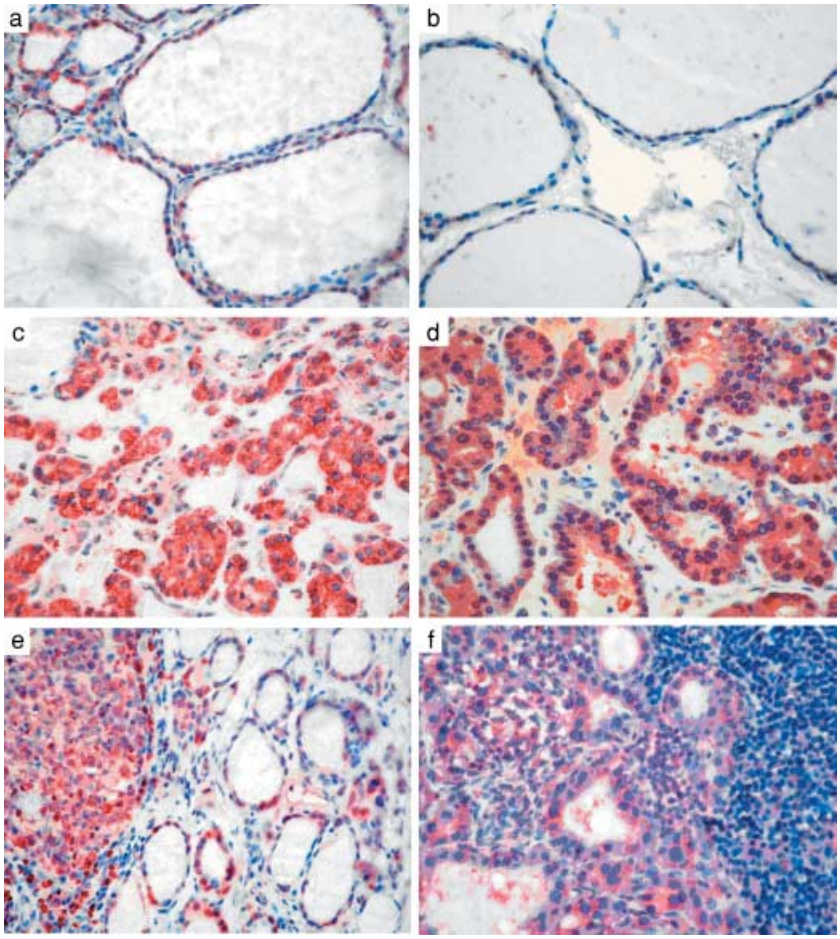

Fig. 1 Immunohistochemical demonstration of NOS2 (a, c and e) and NOS3 (b, d and f) in normal thyroid (a and b), papillary thyroid carcinoma (PTC) (c and d) and Hashimoto's thyroiditis (e and f). Note the intense red labelling of NOS2 and NOS3 in PTC.

$r = 0{\cdot}89$); angiopoietin-2, forward 5′-GGCAGCTTGTTTTTCT-TCGCT-3′, reverse 5′-CTGATACTGCCTCTTCCCCG-3′ (slope −3·04, $r = 0{\cdot}91$, NM_213808); Tie2, forward 5′-TGAGCCTTACTTT-GGGGATG-3′, reverse 5′-TGCACGCAGAGCTCATATTC-3′ (slope −2·89, $r = 0{\cdot}92$, AF251494).

Statistics

Data are presented as mean ± SEM. Differences between groups were assessed by Kruskal–Wallis and Mann–Whitney tests. Correlations within each group were sought using Spearman's method. Data for the ET-1 mRNA levels reported previously in the same patients[3] were used to assess the correlations between the ET-1 axis, on the one hand, and NO synthases, VEGF, angiopoietin-1 and -2 on the other. A P-value of $< 0{\cdot}05$ was considered to be statistically significant.

Results

Morphometry

In normal thyroid, papillary carcinoma and Hashimoto's thyroiditis, the areas occupied by follicular cells were, respectively, 11·3 ± 0·9%, 77·8 ± 4·4% and 5·9 ± 1·0% whereas the areas occupied by inflammatory cells were 0·06 ± 0·03%, 0·8 ± 0·3% and 58·2 ± 5·8% in the same groups, respectively.

Endothelin-1

As reported previously,[3] the percentages of ET-1-positive follicular cells were, respectively, 37·0 ± 6·5%, 96·8 ± 5·1% and 71·6 ± 10·7% in normal thyroid, PTC and Hashimoto's thyroiditis. The values observed in PTC and in Hashimoto's thyroiditis were significantly greater ($P < 0{\cdot}001$) than in normal thyroid. RTQ-PCR also revealed that ET-1 mRNA levels were, respectively, 3·8 ± 1·3 and 3·0 ± 0·5 times greater ($P < 0{\cdot}001$) in PTC and Hashimoto's thyroiditis than in normal thyroid.

NO synthases

The results of the immunohistochemistry are illustrated in Fig. 1. NOS2 immunoreactivity was detected in the cytoplasm of follicular cells but also in inflammatory cells of Hashimoto's thyroiditis, which massively invaded the thyroid tissue. In normal thyroid, in Hashimoto's thyroiditis and in PTC, the percentages of NOS2-positive follicular cells were, respectively, 67·3 ± 5·2% (range 56·8–86·5), 68·1 ± 3·7% (range 56·5–77·7) and 96·6 ± 2·0% (range 87·5–99·8). Values were significantly higher in thyroid carcinoma ($P < 0{\cdot}005$) than in normal thyroid and Hashimoto's thyroiditis. As shown in Table 1, the total score of NOS2 immunostaining was also greater in PTC than in normal thyroid ($P < 0{\cdot}05$) and Hashimoto's thyroiditis ($P < 0{\cdot}005$). NOS3 immunoreactivity was found in the cytoplasm of

Table 1. Scores for NOS2 and NOS3 immunostaining

	Weak	Strong	Total
NOS2			
Normal thyroid	43 ± 5 (25–51)	48 ± 19 (17–122)	91 ± 15 (69–148)
Papillary thyroid carcinoma	42 ± 16 (1–97)	93 ± 27 (5–190)	151 ± 16*,** (102–199)
Hashimoto's thyroiditis	48 ± 3 (43–60)	39 ± 7 (17–56)	88 ± 6 (65–99)
NOS3			
Normal thyroid	42 ± 2 (37–47)	61 ± 17 (37–109)	103 ± 16 (84–149)
Papillary thyroid carcinoma	16 ± 10 (0–52)	159 ± 25 (66–200)	174 ± 17* (105–200)
Hashimoto's thyroiditis	44 ± 4 (36–59)	92 ± 10 (60–112)	136 ± 6 (119–150)

The results are expressed as mean ± SEM (range).
*$P < 0{\cdot}05$ *vs.* normal; **$P < 0{\cdot}005$ *vs.* Hashimoto's thyroiditis.

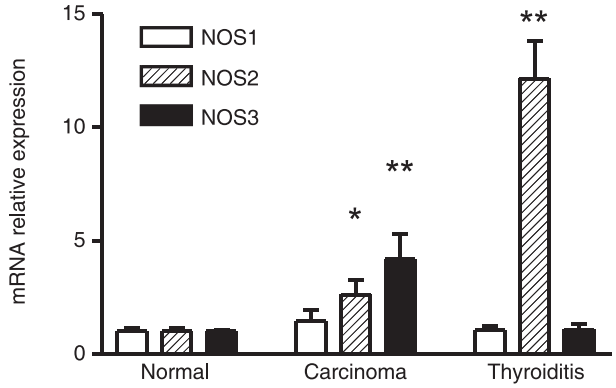

Fig. 2 mRNA relative expression of NOS1, NOS2 and NOS3 determined by RTQ-PCR in normal thyroid, papillary thyroid carcinoma and thyroiditis. Note the overexpression of NOS2 and NOS3 in carcinoma and of NOS2 in thyroiditis. The results are expressed as mean ± SEM. *$P < 0{\cdot}02$, **$P < 0{\cdot}005$ *vs.* normal.

follicular cells as well as in endothelial cells in thyroid carcinoma. In Hashimoto's thyroiditis, NOS3 expression was observed in follicular cells, endothelial cells and some inflammatory cells. However, NOS3 immunostaining in lymphocytic cells was sparse, much weaker than that of NOS2 and extremely variable from case to case or even from one cell group to another in the same case. No area was entirely positive or negative. NOS3 was expressed in 94·9 ± 4·7% (range 71·5–100%) and 90·1 ± 1·8% (range 85·8–96·2%) of follicular cells in PTC and thyroiditis, respectively, whereas it was expressed in only 72·6 ± 7·4% of normal thyroid cells. NOS3 expression was greater than normal in PTC ($P < 0{\cdot}02$) but did not differ significantly in Hashimoto's thyroiditis compared with that observed in normal and papillary carcinoma. The score for NOS3 immunostaining was also increased in PTC compared with normal thyroid ($P < 0{\cdot}05$).

As shown in Fig. 2, the results of RTQ-PCR revealed that NOS2 mRNA levels were, respectively, 2·6 ± 0·6 and 12·1 ± 1·7 times greater in PTC ($P < 0{\cdot}02$) and in Hashimoto's thyroiditis ($P < 0{\cdot}005$) than in normal thyroid. NOS2 mRNA levels were also higher in Hashimoto's thyroiditis than in PTC ($P < 0{\cdot}0001$). However, NOS3 was only overexpressed in PTC ($P < 0{\cdot}005$ *vs.* normal thyroid and Hashimoto's thyroiditis). No difference in NOS1 expression was found between the three groups.

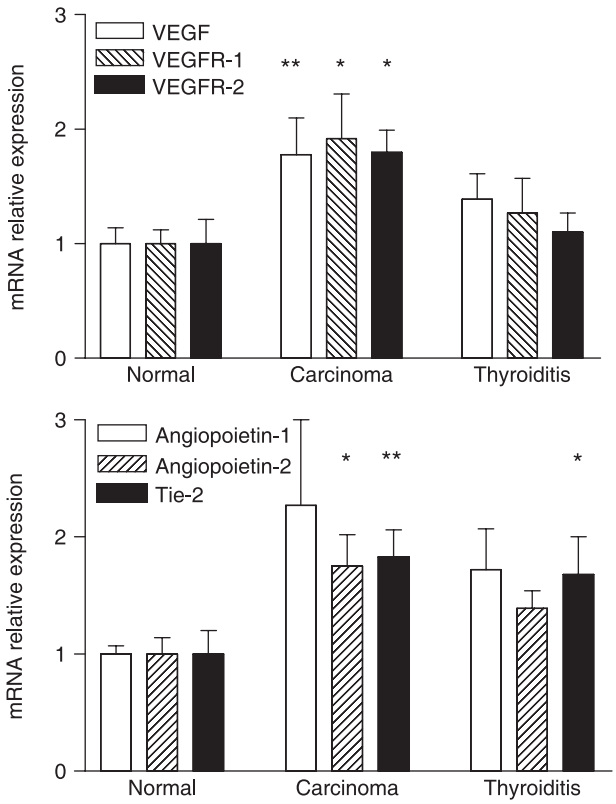

Fig. 3 mRNA relative expression of VEGF, VEGF receptors (VEGFR-1 and -2), angiopoietin-1, angiopoietin-2 and angiopoietin receptor (Tie2) determined by RTQ-PCR in normal thyroid, thyroid papillary carcinoma and Hashimoto's thyroiditis. Note the overexpression of VEGF, VEGFR-1, VEGFR-2, angiopoietin-2 and Tie2 in carcinoma and of Tie2 in thyroiditis. The results are expressed as mean ± SEM. *$P < 0{\cdot}05$; ** $P < 0{\cdot}02$ *vs.* normal.

VEGF, angiopoietins and their receptors (Fig. 3)

VEGF mRNA levels were increased by 1·8 ± 0·3 times ($P < 0{\cdot}02$) in PTC compared with normal thyroid. Regarding VEGF receptors VEGFR-1 and VEGFR-2, mRNA levels were, respectively, 1·9 ± 0·4 and 1·8 ± 0·2 times greater in PTC than in normal thyroid ($P < 0{\cdot}05$). Expressions of VEGF and its receptors were not different between Hashimoto's thyroiditis and normal thyroid.

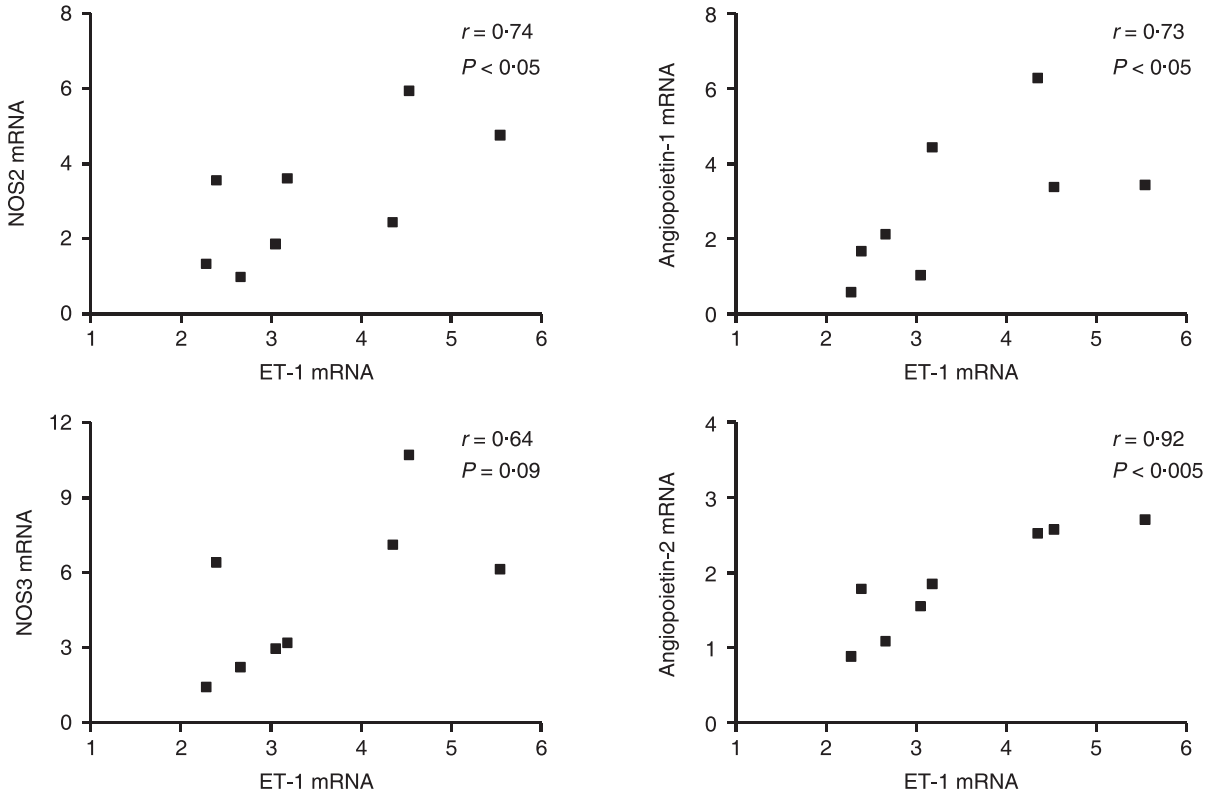

Fig. 4 Correlations between ET-1 mRNA levels and NOS2, NOS3, angiopoietin-1 and -2 mRNA levels in papillary thyroid carcinoma. Correlations were performed using Spearman correlation tests (*r* corresponds to the Spearman correlation coefficient).

Despite a tendency for angiopoietin-1 mRNA to display higher levels than normal in PTC, the difference did not reach significance because of a large SEM ($P = 0{\cdot}09$). However, mRNA levels of angiopoietin-2 and its Tie2 receptor, were significantly different ($P < 0{\cdot}05$) between groups. Indeed, overexpression of angiopoietin-2 and Tie2 was observed in PTC ($P < 0{\cdot}05$) compared with normal. Regarding Hashimoto's thyroiditis, only Tie2 was significantly overexpressed ($P < 0{\cdot}05$ *vs.* normal).

Correlations between ET-1 mRNA levels and mRNA levels of NOS and angiogenic factors (Fig. 4)

In PTC, close correlations were found between the expressions of ET-1 mRNA and those of NOS2 ($P < 0{\cdot}05$) whereas the correlation with NOS3 just failed to reach significance ($P = 0{\cdot}09$). ET-1 mRNA levels also correlated with those of angiopoietin-1 ($P < 0{\cdot}05$) and angiopoietin-2 ($P < 0{\cdot}005$) but not with VEGF. Of note, no correlation was observed between ET-1 mRNA levels and the above parameters in Hashimoto's thyroiditis. Expression of ET-1 receptors (ET_A and ET_B) did not correlate either with the expression of angiogenic factors.

Other correlations (Fig. 5)

In PTC, NOS2 mRNA levels correlated with those of NOS3 ($r = 0{\cdot}76$, $P < 0{\cdot}05$) and those of angiopoietin-2 ($r = 0{\cdot}88$, $P < 0{\cdot}01$). However, NOS2 mRNA did not correlate with VEGF and angiopoietin-1 mRNAs. In Hashimoto's thyroiditis, NOS2 mRNA tended to correlate with NOS3 and angiopoietin-2 without reaching significance ($r = 0{\cdot}65$; $P = 0{\cdot}066$). NOS3 expression correlated with that of angiopoietin-2 ($P < 0{\cdot}05$) in PTC and Hashimoto's thyroiditis. In summary, there was a significant correlation between mRNA levels of NOS2 and NOS3 and those of angiopoietin-2 (Fig. 5).

Discussion

The present study demonstrates by immunohistochemistry and RTQ-PCR enhanced expression of NOS2 (inducible) and NOS3 (endothelial) in PTC compared with normal thyroid whereas expression of NOS1 (neuronal) was unchanged. In Hashimoto's thyroiditis, RTQ-PCR revealed that only NOS2 was overexpressed and this occurred to a much greater extent than in thyroid carcinomas. On the basis of the immunohistochemical findings, this higher NOS2 expression observed in RTQ-PCR could be mainly due to inflammatory cells. This is not surprising in view of the role of NOS2 in immunomediated processes and its production by inflammatory cells. Of note, a lymphocytic component did not influence our results of RTQ-PCR in PTC because those samples contained very few lymphocytes, as reflected by the morphometric study. Regarding malignant lesions, previous authors found in breast cancers that NOS2 was not only expressed in stromal cells and tumoral macrophages but also in the tumour cells themselves.[21,22] Further studies also demonstrated NOS2 expression by the cells of other cancers, such as those arising in the ovary,[8] oesophagus,[23] colon[23] and prostate.[24]

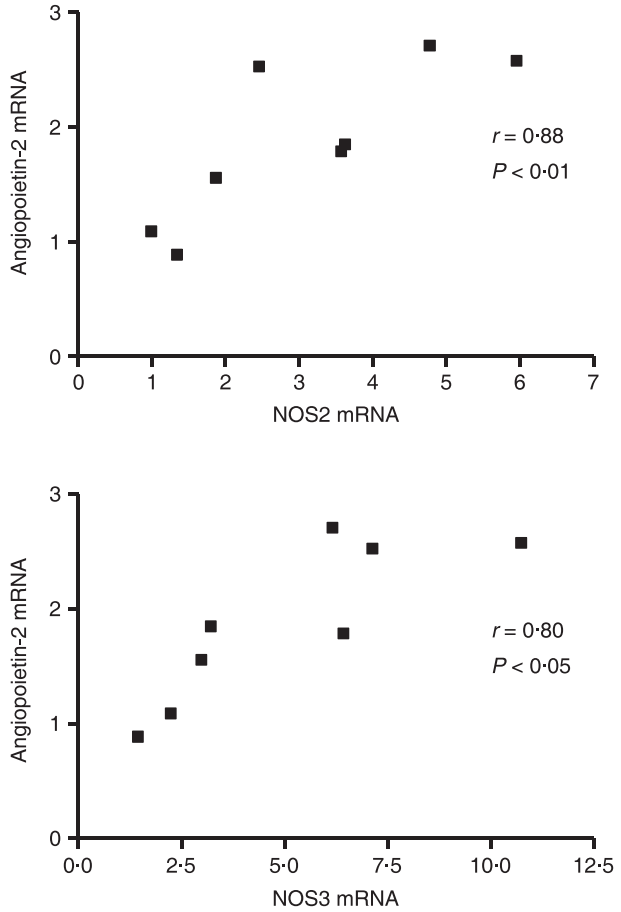

Fig. 5 Correlations between mRNA levels of NOS2 and NOS3 and angiopoietin-2 mRNA level in papillary thyroid carcinoma. *r* corresponds to the Spearman correlation coefficient.

In thyroid carcinoma, we now show that NOS2 is also expressed by tumour cells. These results are in line with previous studies showing immunohistochemical staining for NOS2 in PTC.[13,14] These studies, however, used qualitative or semiquantitative approaches. In the present study, we demonstrated conclusively that NOS2 was statistically overexpressed at the levels of its mRNAs and the NOS proteins (as assessed by immunohistochemistry) in PTC compared with normal thyroid. Studying the expression at these two levels is of importance because previous studies have shown little correlation between NOS2 mRNA expression and enzyme activity, suggesting a considerable degree of post-transcriptional regulation.[23] In the 'in situ' ductal breast carcinoma, other authors found a strong coexpression of NOS2 and NOS3.[11] In that cancer, NOS3 was expressed by tumour cells that also expressed oestrogen receptors.[11] In our study, we also found a NOS3 coexpression that was enhanced in thyroid carcinoma compared with normal, as well as a strong correlation between NOS2 and NOS3 mRNAs. This raises the question of the respective roles of NOS2 and NOS3 in tumorigenesis. In contrast to NOS3, which produces a trace amount of NO to mediate physiological functions, NOS2, readily up-regulated in inflammatory or infectious conditions, generates large amounts of NO (μM), sometimes for hours to days. It has been suggested that the amount of NO produced in cancer could exert opposite effects.[7,11] Indeed, high concentrations of NO, such as those produced by macrophages, could mediate cancer cell apoptosis and an inhibition of tumour growth whereas low concentrations of NO, similar with those measured in cancer samples, could promote cancer growth, proliferation and new blood vessel formation.[25] From our study, however, we cannot draw definite conclusions about the role of NO in thyroid cancer because this question was not addressed specifically.

There is a now evidence that ET-1 may play a role in tumorigenesis by modulating mitogenesis, apoptosis, angiogenesis and development of metastases.[6] Our previous studies demonstrated an overexpression of the ET-1 axis in PTC.[3–5] In the present study, we show that ET-1 expression is coordinated with that of NOS. The reciprocal interactions between ET-1 and NO have been well recognized in cardiovascular physiology, a correct balance between the two systems being crucial for the maintenance of cardiovascular homeostasis. ET-1 has been shown to enhance NO production through ET_B receptors located on endothelial cells.[26] Conversely, NO can inhibit ET-1 production.[27] ET-1 is also a proinflammatory cytokine by itself or by stimulating monocytes to produce cytokines, which could induce NOS2.[28] The correlation observed between ET-1 and NOS2 in thyroid cancer is therefore not surprising.

The last important finding of our study was the evidence of an overexpression of angiogenic factors and their receptors in PTC. VEGF has been previously shown to be present in epithelial cells of normal thyroid, goitre, Graves' disease, thyroiditis and thyroid tumours.[29] Although not a specific marker of malignancy in the thyroid gland, increased VEGF expression has been correlated with more aggressive thyroid tumour behaviour and metastasis.[29,30] Both ET-1 and NO are reportedly able to up-regulate VEGF expression.[16,18] However, our study failed to disclose a correlation between VEGF and ET-1 or NOS expressions. In other cancers, such as breast carcinoma, a correlation was found between ET-1 or its receptors and VEGF expression.[17] Nevertheless, our results also showed that VEGF receptors (VEGFR-1 and -2) were overexpressed in thyroid carcinoma (Fig. 3).

However, because RTQ-PCR analyses mixed cellular populations, our study was unable to distinguish which cell types (follicular or endothelial cells) were responsible for this overexpression. Previous studies have demonstrated an expression of these receptors in PTC by either semiquantitative RT-PCR analysis[31] or immunohistochemistry.[30] In the latter study,[30] VEGFR-1 immunostaining was restricted to endothelial cells with a patchy distribution in carcinoma. It is noteworthy that VEGFR-1 expression may not be restricted to endothelial cells as it was found to occur in rat thyroid epithelial cells, implying a role for VEGF in thyroid cell growth and function.[32] Other authors also supported the view of a nonendothelial expression of the other VEGF receptor by detecting a VEGFR-2 immunopositivity in rat adenohypophysial cells.[33] Finally, in the ovarian carcinoma, an expression of both VEGF receptors was evidenced in ascitic tumour cells that coexpressed VEGF.[34] VEGFR-2 was even detected in tumour cells at primary malignant sites.

In our study, we also found a clear overexpression of angiopoietin-2 and Tie2 (Fig. 3). There was a tendency for angiopoietin-1 mRNA levels to increase without reaching statistical significance. Among angiogenic factors, angiopoietin-1 and -2 play important roles in

various aspects of angiogenesis. After initiation of blood vessel formation by VEGF, angiopoietin-1 binding to the tyrosine-kinase receptor Tie2 has been shown to stabilize newly formed blood vessels through recruitment and interaction with pericytes.[19,31] Of note, angiopoietin-1 can also offset VEGF-induced angiogenesis.[20] Conversely, angiopoietin-2, by competitively binding to Tie2, antagonizes the function of angiopoietin-1 and destabilizes the vessel to facilitate VEGF-induced angiogenesis and the formation of capillary sprouts.[19] Our results related to the angiopoietin system are in agreement with another study showing a marked up-regulation of angiopoietin-2 that, in association with high VEGF levels, correlated with tumour size of thyroid neoplasms.[31] A moderate up-regulation of Tie2 was also observed in a few PTCs and in most medullary thyroid carcinomas.[31] However, angiopoietin-1 mRNA expression was slightly down- or up-regulated in a few carcinoma. Our study (Fig. 4) disclosed correlations between ET-1 expression and those of angiopoietin-1 and -2. The possible effect of ET-1 on angiopoietin expression should be questioned, but to our knowledge has never been evaluated. Regarding the NO pathway, we found that expressions of NOS2 and NOS3 correlated with those of angiopoietin-2 (Fig. 5). The relationship between NO and angiopoietin certainly deserves further investigation, considering a recent study showing that endothelial-derived NO is required for angiogenic actions of angiopoietin-1.[35]

In conclusion, the present study provides a global view of an interrelated system that encompasses NO, ET-1 and angiogenic factors and is up-regulated in thyroid carcinoma. Each of its components could play a role in the pathogenesis of cancer. Further studies are now required to identify the key targets in this system for future anticancer therapy.

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