Nitric Oxide Synthase Activity in Human Thyroid Papillary Carcinomas

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In human thyroid carcinoma, there is no evidence that the activity of NO synthase and the contribution of endothelial NOS (eNOS) have been determined. To determine the relationship between NOS and human thyroid carcinomas, we assayed eNOS expression and assessed the activity of NOS in thyroid papillary carcinomas (TPC), benign thyroid tumors, and normal thyroid tissues. We investigated the involvement of NO in TPC using an assay system to measure NOS activity by high performance liquid chromatography (HPLC), which could be applied to determine citrulline enrichment. The TPC showed high levels of NOS activity. Moreover, histochemical staining with

NADPH-diaphorase revealed a positive reaction in the TPC samples, localized to the blood vessel endothelium. These cells were much less stained, however, in normal thyroid tissue. When we incubated these samples with an anti-eNOS antibody, we observed staining of the TPC samples, again localized to the blood vessel endothelium cells, but much less than in the normal thyroid samples. RT-PCR amplification using eNOS specific primers revealed that eNOS mRNA was abundant in the TPC samples, but was much less expressed in normal thyroid. These results show that NOS activity exists in TPC and that it is associated with malignancy.

Key words: HPLC, eNOS, iNOS, Thyroid, Papillary carcinoma

I. Introduction

Nitric oxide synthase (NOS) is an enzyme that synthesizes nitric oxide in cells. Three isoforms of this enzyme, neural (nNOS), endothelial (eNOS) and macrophage (mNOS), have been cloned and sequenced. Functionally, it has been shown that the first two isoforms are a constitutive (cNOS) type and the last one is an inducible (iNOS) type. Nitric oxide (NO) may be involved in the endogenous formation of carcinogenic nitrosamines, as well as in the deamination of DNA nucleobases [13]. NO has also been shown to be related to angiogenesis, which occurs not only during the reproductive cycle and wound healing, but also during tumorigenesis [14]. Excess NO produced in inflamed tissues may play an additional role in carcinogenesis by impairing the tumor suppressor function of p53 [3]. eNOS is expressed in human thyroid tissues. The endothelial localization of eNOS is consistent with the role of NO in the vascular con-

trol of the thyroid. eNOS expression in thyroid follicular cells and the variations in its immunoreactivity suggest a possible role for NO in thyrocyte function and/or growth [5, 6, 16]. iNOS is present in monocytes from patients with Graves' disease [11]. Several reports in animals suggest a tumoricidal role for NO, but in human tumors its role has not been well-established. We reported previously that iNOS was expressed in the human thyroid papillary carcinoma, and also that iNOS was mostly derived from tumor cells rather than macrophages using the RT-PCR method and the immunohistochemical method [9]. In the present study, we assayed eNOS expression and compared the activity of NO synthase in human thyroid papillary carcinomas, benign thyroid tumors, and normal thyroid tissues to determine whether any relationship exists between NO and carcinogenesis in the human thyroid.

II. Materials and Methods

Tissues specimens

Human thyroid papillary carcinomas (TPC) were obtained at the time of surgery from 5 patients. Samples

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Table 1. Study subjects.

Case	age	sex	tumor type
1	43	F	papillary carcinoma
2	55	F	papillary carcinoma
3	59	F	papillary carcinoma
4	66	F	papillary carcinoma
5	69	M	papillary carcinoma
6	69	F	follicular adenoma
7	61	F	follicular adenoma
8	51	F	follicular adenoma
9	46	M	follicular adenoma
10	68	F	adenomatous goiter

did not include necrotic tissue. Benign thyroid tumors were obtained from 4 cases of follicular adenoma and a case of adenomatous goiter patients (Table 1). Five normal thyroid glands from the adjacent tissue to the tumor tissue were treated as a control group. A part of the sample was placed immediately into the cold medium and prepared for subsequent assay for NO synthase activity. The rest of the sample was frozen in liquid nitrogen as soon as possible and stored at -80°C for subsequent analysis.

Assay of NOS activity

In order to assess NOS activity of a tissue, we measured the citrulline amount in the tissue. Tissues were incubated in 5 ml of Ringer–Locke solution with or without 0.1 mM L-NA maintained at $37\pm 0.3^{\circ}\text{C}$ and aerated with a mixture of 95% O_2 and 5% CO_2 . Constituents of the solution were as follows: 120 mM NaCl, 5.5 mM KCl, 2.2 mM CaCl_2 , 1.0 mM MgCl_2 , 25.0 mM NaHCO_3 and 5.6 mM glucose. 50 μl of the bathing medium was sampled every 3 min. The sample was deproteinized by ultrafiltration (Microcon YM-10, Amicon, Beverly, USA). The filtrate was kept in a freezer until the time of assay. O-phthalaldehyde (OPA; Nacalai Tesque, Kyoto, Japan) reagent was prepared by mixing 10 mg of OPA, 500 μl ethanol, 9.5 ml of 0.4 M sodium borate buffer pH 9.5, and 10 μl of 2-mercaptoethanol. 10 μl of the sample was mixed with 20 μl of the OPA reagent for 60 sec. 25 μl of the mixture was injected into high performance liquid chromatography system (LC-10AS, SCL-10A, C-R4A, Shimadzu, Kyoto, Japan) coupled to an octadecylsilane column (4.6 $\times$ 250 mm, Nacalai Tesque, Kyoto, Japan) and a fluorescence detector (RF-10A, Shimadzu, Kyoto, Japan). The excitation and detection wave length were set to 340 and 455 nm, respectively. The mobile phase was 0.1 M sodium citrate buffer pH 6.0, and a linear gradient of 25 to 85% methanol was used. The flow rate was 0.8 ml/min. The difference of citrulline amount with and without L-NA in the same tissue was considered as NOS activity.

NADPH-diaphorase histochemistry and eNOS immunohistochemistry

Tissues were immersed for 2 hr at 37°C in 0.1 M phosphate-buffer saline (PBS) containing 10% gelatin. Each specimen was cut into 5 μm or 20 μm -sections thick. For

NADPH diaphorase histochemistry, tissue sections were washed in PBS and immersed for 1 hr in a reagent mixture (5 mg/ml NADPH, 5% NBT 0.1% Triton in PBS, pH 8). For immunohistochemistry, 20 μm tissue sections were washed in 0.1 M PBS-Triton solution and incubated with a 1:5000 dilution of anti-eNOS monoclonal antibody (Transduction Lab, Lexington, KY USA) for 48 hr at 4°C . Sections were washed thoroughly in PBS and incubated for 1 hr with rabbit anti-mouse IgG (Vector laboratories, Burlingame, CA USA). After further washing, the sections were incubated for 1 hr with a 1:4000 dilution of avidin-biotin-peroxidase complex (Vectastain Elite ABC Kit, Vector laboratories). Following treatment with diaminobenzidine-nickel, the sections were examined under a light microscope.

Preparation of RNA and RT-PCR

Total RNA was isolated from the TPC and normal thyroid specimens by the acid guanidium thiocyanate-phenol method [3]. Each 5 μg of total RNA was treated with 10 units DNase (Pharmacia, Piscataway, NJ USA) and 20 units of RNasin (Wako Pure Chemicals, Tokyo Japan) for 1 hr at 37°C to eliminate trace DNA contamination. The DNase was inactivated by heat for 5 min at 95°C and was removed by extraction with phenol:chloroform:isoamyl alcohol (24:24:1), and the RNA was precipitated with ethanol. cDNA was synthesized from each RNA sample using 800 units reverse transcriptase (Life technologie, Frederick, MD USA) and 500 pmol of oligo-dT_{12–18} (Pharmacia). The transcriptase was subsequently heat inactivated for 10 min at 65°C .

PCR

The PCR mixture for eNOS cDNA consisted of 1XPCR buffer (Takara Japan), 4.0 mM MgCl_2 , 0.2 μM dNTPS, 2.5 units Taq DNA Polymerase (Takara), 10 $\mu\text{g}/\text{ml}$ cDNA template and the human eNOS cDNA primers, 5'-GCCTTTGCTCGTGCCGTGGACA-3' (sense, corresponding to nt 2004–2025) and 5'-CCCTCGTGGAAGTCTGCTGCTTTC-3' (antisense, corresponding to nt 2349–2328), which yielded a product of 346 bp. PCR was performed in an Iwaki TSR-300 thermal cycler, following, preincubation at 94°C for 1 min, amplification 38 cycles (30 sec of denaturation at 93°C , 40 sec of annealing at 59°C , 1 min of extension at 72°C), followed by a 6 min extension at 72°C . β -actin cDNA was simultaneously amplified according to the same protocol, using the primers, 5'-TGGTGGGTATGGGTCA-GAAGGACTC-3' (sense) and 5'-CATGGCTGGGGTGT-TGAAGGTCTCA-3' (antisense), which yielded a product of 266 bp [12]. A water blank was used as a negative control in all experiments.

PCR products were electrophoresed in 1% NuSieve/1% Seakem (FMC Products, Rockland, ME USA). DNA bands were cut out and the DNA in each band was eluted from the gel using a Gene Clean II kit (Bio 101, Vista, CA USA) followed by ethanol precipitation. The DNA was cloned into the PCR II (Invitrogen, Carlsbad, CA USA) using a DNA ligation kit (Invitrogen). The resulting plasmid was used to

Fig. 1. Citrulline contents in human thyroid. NO synthase activity was determined by a difference of citrulline amounts in the presence and absence of L-NA, a specific NOS inhibitor. Results are means $\pm$ S.E. of 5 samples. Significantly different from the value with TPC, * $P < 0.05$, ** $P < 0.01$. NS: not significant. TPC: thyroid papillary carcinoma, 5 cases; Benign thyroid tumor: follicular adenoma, 4 cases and adenomatous goiter, 1 case; Normal thyroid: normal thyroid, 5 cases.

transform *E. coli* using the heat shock method and the bacteria were grown according to standard procedures. Plasmid DNA was extracted from the bacteria by alkaline lysis, purified and applied to a DNA sequencer (A. L. F., Pharmacia).

Statistical analysis

Data are presented as mean $\pm$ standard error of the mean (SE), and comparisons between groups were performed using Student's *t* test. *P* values less than 0.05 are considered to be significant.

III. Results

Nitric oxide synthase activity

Citrulline formation was increased significantly in TPC compared with that in benign thyroid tumors. Moreover, this increase was completely abolished in the presence of 0.1 mM L-NA. These results indicate that NO synthase activity is present and functioning in TPC. On the other hand, the NOS activity was not detectable in benign thyroid tumors and in normal thyroid tissues (Table 2). Citrulline formation in normal thyroid was significantly less than in TPC and benign thyroid tumor (Fig. 1).

Histochemical localization of NADPH-diaphorase

In the human normal thyroid, positive stainings indicated by a blue reaction product were observed in some follicular cells and vascular endothelial cells (Fig. 2, left). Also, the staining of NADPH-diaphorase in the endothelial cells was seen in human papillary carcinomas (Fig. 2, right), which was stronger than that in normal tissues.

Immunohistochemical detection of eNOS

Incubation of normal thyroid and TPC samples with an anti-eNOS monoclonal antibody revealed a positive immu-

nohistochemical reaction, indicated by the presence of a blue reaction product in the TPC samples. This staining was localized to the endothelial cells of the blood vessels (Fig. 3, right). In the normal thyroid, positive stainings were observed in the endothelial cells of blood vessels (Fig. 3, left), but they were much less compared to cells of tumor tissues.

RT-PCR detection of eNOS mRNA

When we utilized human eNOS primers to amplify sequences in human TPC cDNA, we observed a band of the expected size, as well as two additional bands (Fig. 4). Cloning and sequencing of the PCR products showed that the 346 bp band correspond to eNOS mRNA, whereas the additional bands correspond to human vimentin mRNA and human

Table 2. Citrulline content in thyroid tissue.

Case	umor type	Citrulline (pmole/min/g)	
		L-NA (-)	L-NA (+)
1	papillary carcinoma	96.3	90
2	papillary carcinoma	38.7	35
3	papillary carcinoma	83.6	24.6
4	papillary carcinoma	74.4	20.7
5	papillary carcinoma	96.6	34.3
6	follicular adenoma	5.7	7.7
7	follicular adenoma	35.6	32.7
8	follicular adenoma	36.1	43.4
9	follicular adenoma	42.3	34.9
10	adenomatous goiter	47.2	42.9
11	normal thyroid	19.9	14.8
12	normal thyroid	9.9	10.9
13	normal thyroid	16.4	16.6
14	normal thyroid	16.8	21.1
15	normal thyroid	4.6	7.8

Fig. 2

Fig. 3

Fig. 4. RT-PCR amplification of an eNOS sequence in human thyroid samples. Primers specific for the eNOS (top panel) and β -actin genes (bottom) were used to amplify sequences in cDNA derived from thyroid carcinomas and normal thyroid tissue. Lane 1, marker DNA (M); lanes 2–4, normal thyroid samples (normal); lanes 5–8, papillary thyroid carcinomas (carcinoma); lane 9, water blank (B).

clone 22530. All three of these bands were present in each of the four thyroid carcinoma specimens, whereas only clone 22530 was amplified in the 3 normal thyroid tissue samples (Fig. 4).

IV. Discussion

It has been suggested that NO plays a dual role in tumor growth behavior, as an agent responsible for cellular death, or as a mediator of tumor cell proliferation [20]. NOS has been shown to have a direct role in human carcinogenesis by catalyzing the production of nitrosamines [13]. A more indirect role for this enzyme may consist of the production in infected and inflamed tissues, which may also contribute to the carcinogenic process [1, 10, 13–15].

We investigated the involvement of nitric oxide in TPC by measuring the citrulline amount with and without a nitric oxide synthase inhibitor, L-NA. The present study showed that high levels of NOS activity were present in TPC, while the enzyme activity was not detectable in benign thyroid tumors and in normal thyroid, suggesting that NOS activity may be related to the malignancy of tumors. Basal content of citrulline in normal tissue was less than that in TPC and benign tumor. The reason is unknown, but the metabolism may be accelerated in the tumor tissue.

In our previous study, we reported that iNOS was ex-

pressed in the TPC and also that iNOS was mostly derived from tumor cells rather than macrophages using the immunohistochemical and RT-PCR methods [9]. It was observed that iNOS activity, as well as NO biosynthesis, was increased in invasive cancer of the human breast and gynecological cancers compared with specimens of benign and normal tissue [18, 19]. These findings directly link iNOS to carcinogenesis. Immunohistochemical investigations showed that in breast cancers, iNOS staining was present mainly within tumor-associated macrophages [19]. However, few tumor-associated macrophages were detected in gynecological cancers; these cells were mainly confirmed to be stroma and did not react with anti-NOS antibody [18]. In addition, iNOS was detected in epithelial cells throughout squamous cell carcinomas of the head and neck, and enzyme activity was highest in the area directly surrounding keratin pearls [2].

In the present study, the NADPH-diaphorase staining and the immunohistochemical staining for eNOS revealed a positive reaction in the TPC samples localized to vascular endothelial cells, but much less in the normal thyroid tissues. RT-PCR amplification using eNOS specific primers revealed that eNOS mRNA was abundant in the TPC samples, but much less in the normal thyroid samples. One may consider that vascular density is higher in tumor tissues than in normal tissues, but this may not be the case, because the

Fig. 2. Detection of NADPH-diaphorase in human thyroid tissues. The presence of a blue reaction denotes the enzyme. In normal thyroid, NADPH-diaphorase was expressed in some follicular cells (arrowhead) and vascular endothelial cells (arrow) (normal, $\times 50$). In TPC, NADPH-diaphorase was found in the vascular endothelial cells (carcinoma, $\times 50$).

Fig. 3. Immunohistochemical localization of eNOS in human thyroid tissues. Thyroid tissue samples were stained with a monoclonal antibody to eNOS; a positive reaction is indicated by a blue color (normal, $\times 50$; carcinoma, $\times 50$).

amount of actin mRNA in the same amount of total RNA applied did not differ in both tissues. An abundant presence of eNOS in the TPC suggests another connection between NOS and carcinogenesis [1]. Since endothelial cell adhesion and vascular permeability are necessary in the early stages of metastasis, eNOS may be important in both primary growth and early stages of metastasis of human thyroid papillary carcinoma.

Vascularization is an absolute requirement for growth of solid tumors and it correlates positively with tumor metastasis. It has recently been shown that the increased tumor growth associated with iNOS-cells correlates with increased angiogenesis [7]. NO has been identified as an important regulator of tumor blood flow in experimental tumors, and inhibition of NO production in these tumors by systemically administered NOS inhibitors decreased tumor blood flow and reduced tumor growth [2, 8]. It is suggested that NO production by nNOS and eNOS in tumor endothelial cells may increase tumor edema and blood supply to the tumor [4]. We provided evidence that in the TPC, iNOS was mostly derived from tumor cells and eNOS highly expressed in tumor endothelial cells. We surmise that in the TPC, iNOS is correlated with angiogenesis in tumor and eNOS is associated with vascular permeability and blood flow to the tumor. It is inferred that these two isoforms are closely related to each other in the tumor growth and metastasis. The evidence for this relationship is not clear, however, and further investigation is needed.

NOS is present in the thyroid tissues and NO may play an important role in the thyroid gland by controlling the blood flow and by directly acting on the thyroid follicular cells [5, 17]. The distribution pattern of NO synthase activity in human thyroid, measured by our biochemical method, was almost consistent with the immunohistochemical data reported. Our work suggests that neoplastic activity in human thyroid papillary carcinoma is associated with NO production, although it cannot be determined which isoform of NOS is more associated with its activity in TPC tissues. These results also suggest that NO may have some important roles in the growth and metastasis of TPC. Further studies are necessary to investigate the effect of NO on malignant tumors.

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VI. References

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