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Nitric Oxide Synthase Activity in Human Lung Cancer

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Nitric oxide synthases (NOS) exist in human tumor cell lines and solid tumor tissues, and it has been suggested that NO may play important roles in growth, progression or metastasis of tumors. We investigated the activity and distribution of NOS in a series of human lung cancer and normal lung tissues. Seventy-two primary lung cancer samples (44 cases of adenocarcinoma, 18 of squamous cell carcinoma, 4 of large cell carcinoma, 2 of small cell carcinoma, 2 of adenosquamous carcinoma, and 2 of carcinoids) and corresponding normal lung samples were obtained from surgically treated patients. In normal lung tissues, little NOS activity was observed with no correlation between the patients' age and NOS activity. The total NOS activities in lung adenocarcinoma samples were significantly higher than those in other types of lung cancers or normal lung samples ($P<0.05$). Analysis by tumor grade of the adenocarcinoma samples revealed no significant difference of NOS activity between grades. TNM classification showed that, although T stage did not correlate with NOS activity, cancer tissues from patients with N2 disease tended to have lower activity than those from patients with N0 or N1 disease. Immunohistochemical studies revealed that the intensity of NOS immunoreactivity correlated with NOS activity. These results suggest that NO may play an important role in the metabolism and behavior of lung cancers, especially adenocarcinoma.

Key words: Nitric oxide — Nitric oxide synthase — Human — Lung cancer

Since the discovery in 1987 that vascular endothelial cells are able to synthesize nitric oxide (NO) from L-arginine,[1] the presence of this biochemical pathway has been thoroughly documented in many other cell types and its biologic relevance has become apparent.[2] This inorganic free radical gas, synthesized by a family of isoenzymes called NO synthase (NOS; EC 1.14.13.39)[3,4] plays an important role as a cell signaling molecule in the vascular, nervous and immune systems.[5,6] Various NOS isozymes, such as endothelial cell NOS (ec-NOS), inducible NOS (i-NOS), and brain NOS (b-NOS) are localized in vascular endothelial cells, macrophages and the neurons of the brain, respectively.[2] Cells in the vascular and central nervous systems possess calcium-dependent NOS (constitutive NOS: c-NOS)[7–9] and produce NO as a signal transduction mechanism. In contrast, i-NOS, which is induced by cytokines and endotoxin, is in most cases calcium-independent and provides sustained release of NO, mediating cytostatic and cytotoxic effects of the immune system.

Recently, it has been reported that NOS can be found in human tumor cell lines or solid tumor tissues. The presence of the calcium-dependent isoform (c-NOS) and cytokine-induced calcium-independent isoform (i-NOS)[10–12] has been detected in various tumor cell lines. Several groups have also reported that some types of solid tumors contain NOS.[13–15] Several pathophysiologic properties important for tumor cell survival and pathology may be mediated by NO.[16] Recent studies have suggested a role of NO in increasing tumor blood flow, edema, and vascular permeability.[17–19] It was speculated that NO may play important roles in growth, progression or metastasis of tumors. However, little work has been performed to determine the presence and characteristics of NOSs in solid tumors and the correlations among NOS activity, clinical manifestations and grade of malignancy. NO is a cytotoxic and cytostatic molecule which is at the same time a very potent vasodilator. Thus, this molecule may play a paradoxical role in tumor development, as in other biochemical systems which have been reported to be regulated by NO.[20]

The aim of the present work is to examine the presence, characteristics and cellular localization of NOS in lung cancer tissues. NOS activity was determined by measuring the conversion of radiolabeled arginine to citrulline, and the correlation between NOS activity and the pathologic features of tumor samples was examined. Moreover, NOS subtypes were determined by immunohistochemistry.

MATERIALS AND METHODS

Materials Three antibodies were used for NOS detection: monoclonal antibody to b-NOS from rat brain (Sigma, St. Louis, MO) which immunoreacts with human b-NOS, monoclonal antibody to human ec-NOS

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(Transduction Laboratories, Lexington, KY) and monoclonal antibody to i-NOS from murine macrophages (Transduction Laboratories), which immunoreacts with human i-NOS. L-[U-^{14}C]Arginine was purchased from Moravek Biochemicals (Brea, CA). Cation exchange resin Bio-Rex 70 was obtained from Bio-Rad (Hercules, CA). Dithiothreitol, EDTA, soybean trypsin inhibitor, *p*-amidinophenylmethanesulfonyl fluoride hydrochloride, leupeptin, aprotinin, and calmodulin were obtained from Sigma. NADPH, flavin adenine dinucleotide, and flavin mononucleotide were purchased from Wako Pure Chemical Industry (Osaka). Trifluoperazine was prepared from Nacalai Tesque (Kyoto). Other chemical agents were of the highest analytical grade commercially available.

Patients and tissues Seventy-two primary lung cancer samples and corresponding normal lung samples at a distance from the cancer were obtained from patients who were treated surgically at Kumamoto University School of Medicine from 1992 to 1996 (46 men and 26 women, average: 63.8±10.2 years old, range: 29 to 78 years old). None of the cancer patients had received chemotherapy or radiation prior to surgery. The patients' characteristics and clinicopathological data are summarized in Table I. Forty-seven patients showed stage I disease, 14 stage II, and 11 stage IIIA. Tumor tissues and normal samples were frozen immediately in liquid nitrogen after resection and stored at −80°C until assay of NOS activity and immunohistochemical studies.

Histopathologic examination of the tumor samples Samples were postfixed for 2 h in 2% paraformaldehyde in 10 m*M* phosphate-buffered saline (PBS), followed by cryoprotection in 10% sucrose, and sectioned at 6 μm. The sections were stained with hematoxylin-eosin (HE). Independent histologic examination of the resected tumor tissues was performed according to the 1982 WHO histological classification[21] by two different pathologists. There were 44 cases of adenocarcinoma, 18 of squamous cell carcinoma, 4 of large cell carcinoma, 2 of small cell carcinoma, 2 of adenosquamous carcinoma, and 2 of carcinoids. All patients underwent detailed postoperative pathologic tumor-node-metastasis (TNM) staging.

Immunohistochemistry with anti-NOS antibodies Immunohistochemical analysis was performed on tumor samples with high NOS activity (7 cases, all adenocarcinoma, 2 men and 5 women, average age 60.4±9.5 years, range 49 to 72 years old) and low NOS activity (9 cases consisting of 6 adenocarcinoma and 3 squamous cell carcinoma, 6 men and 3 women, average age 67.3±5.3 years, range 57 to 75 years old), as summarized in Table II. Sixteen tumor samples and corresponding normal samples were used for anti-NOS antibody stainings. After inhibition of endogenous peroxidase activity by the method of Isobe *et al.*,[22] slide-mounted tissue sections (6 μm thick) were incubated for 1 h with normal sheep serum at a dilution of 1 : 20, and covered for 1 h with 5 μg/ml of monoclonal anti-b-NOS, anti-ec-NOS or anti-i-NOS antibody. They were washed with 10 m*M* PBS, pH 7.4, and incubated with species-specific sheep anti-mouse immunoglobulin ($F(ab')_2$) conjugated with horseradish peroxidase for 1 h. Peroxidase activity was visualized using 3,3′-diaminobenzidine as substrate. The immunoreactivity was evaluated in a blind fashion by two independent observers. A grade of 0 was assigned to tumors with no detectable signal and grades of 1, 2, and 3 were assigned to tumors with light, moderate, and intense reactivity, respectively. As positive controls for b-NOS, ec-NOS and i-NOS, we used granular cells in the cerebellum of rat, vascular endothelial cells and macrophages in human normal lung samples, respectively.

Tumor samples with relatively high i-NOS activity were immunohistochemically analyzed using cell-type specific monoclonal antibodies; EBM-11 (anti-CD68, DAKO, Glostrup, Denmark) for macrophages, L26 (anti-CD20, DAKO) for B-lymphocytes, and UCHL-1 (anti-CD45R0, DAKO) for T-lymphocytes.

Table I. Clinical Characteristics of Patients with Lung Cancer

No. of patients	72
Sex	
Male	46
Female	26
Age	
Mean	63.8
Minimum	29
Maximum	78
Histology	
Adenocarcinoma	44
Squamous cell carcinoma	18
Other types of cancers	
large cell carcinoma	4
small cell carcinoma	2
adenosquamous carcinoma	2
carcinoid tumor	2
Stage	
I	47
II	14
IIIA	11
Degree of differentiation	
Adenocarcinoma	
well	20
moderate	15
poor	9
Squamous cell carcinoma	
well	4
moderate	10
poor	4

Table II. Summary of Immunohistochemical Studies

NOS activity	Tumor type	b-NOS	ec-NOS	i-NOS
>40 pmol/min/g tissue	P/D adenocarcinoma	3+	0	1+
	P/D adenocarcinoma	2+	0	1+
	M/D adenocarcinoma	1+	3+	0
	M/D adenocarcinoma	2+	0	0
	M/D adenocarcinoma	2+	0	0
	W/D adenocarcinoma	3+	0	1+
	W/D adenocarcinoma	2+	0	0
<20 pmol/min/g tissue	P/D adenocarcinoma	0	0	1+
	P/D adenocarcinoma	1+	0	0
	M/D adenocarcinoma	1+	0	0
	M/D adenocarcinoma	1+	1+	2+
	W/D adenocarcinoma	1+	0	0
	W/D adenocarcinoma	2+	2+	0
	P/D squamous cell carcinoma	2+	0	1+
	M/D squamous cell carcinoma	1+	0	2+
	W/D squamous cell carcinoma	1+	0	1+

The numbers indicate the intensity of the immunoreactivity of the samples: 3+, intense; 2+, moderate; 1+, light; 0, undetectable. P/D, poorly differentiated; M/D, moderately differentiated; W/D, well differentiated.

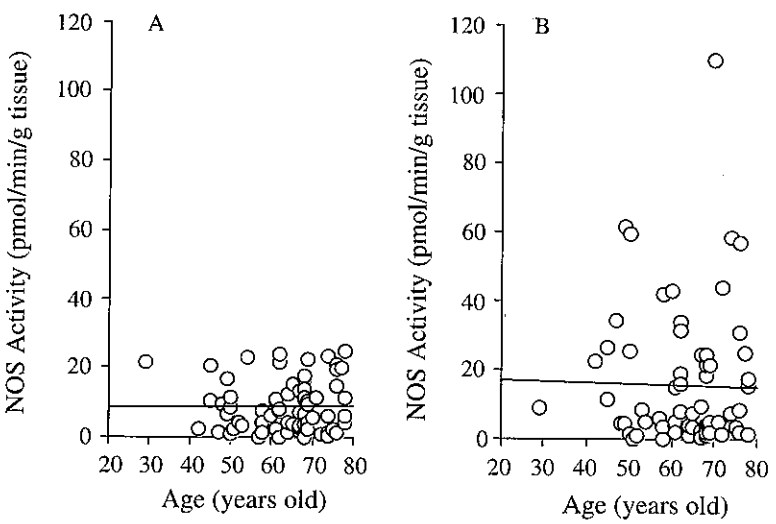

Fig. 1. Correlation between patients' age and total NOS activity in normal lung and cancer samples. Total NOS activities of 72 normal lung and lung cancer samples were assayed as described in the text. A, Normal lung tissues, and B, cancer samples. No significant correlation was observed between the patients' age and NOS activity in either normal or tumor-derived samples.

Assay of NOS activity NOS activity was measured by monitoring the conversion of L-[U-^{14}C]arginine to L-[U-^{14}C]citrulline with the modified method of Bredt and Snyder.[23] The frozen tissues were homogenized at 4°C in 4 volumes (relative to the sample weight) of buffer containing 0.1 m*M* EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 100 μ*M* *p*-amidinophenylmethanesulfonyl fluoride hydrochloride, 1 m*M* dithiothreitol, 0.32 *M* sucrose, and 15 m*M* HEPES, pH 7.6. The homogenates were centrifuged at 100,000*g* at 4°C for 1 h. Supernatants were used for the NOS assay. NOS activity in these supernatants was measured by monitoring the conversion of L-[U-^{14}C]arginine to L-[U-^{14}C]citrulline, using the sodium form of the resin column which absorbs L-[U-^{14}C]arginine. L-[U-^{14}C]Citrulline was eluted and its radioactivity was measured to

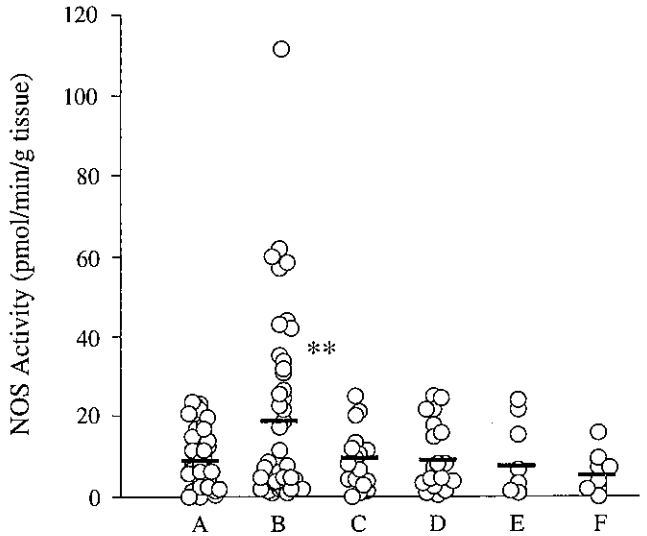

Fig. 2. Total NOS activity in various types of lung cancer. Total NOS activities in 72 lung cancer samples were assayed as described in the text. NOS activity of adenocarcinoma samples was significantly higher than those in the other sample groups. No significant differences were observed among squamous cell carcinoma samples, others (small cell, large cell, adenosquamous and carcinoid tumor), and control samples. A, Paired control samples from adenocarcinoma; B, adenocarcinoma samples; C, paired control samples from squamous cell carcinoma; D, squamous cell carcinoma samples; E, paired control samples of others; F, others. ** $P<0.05$.

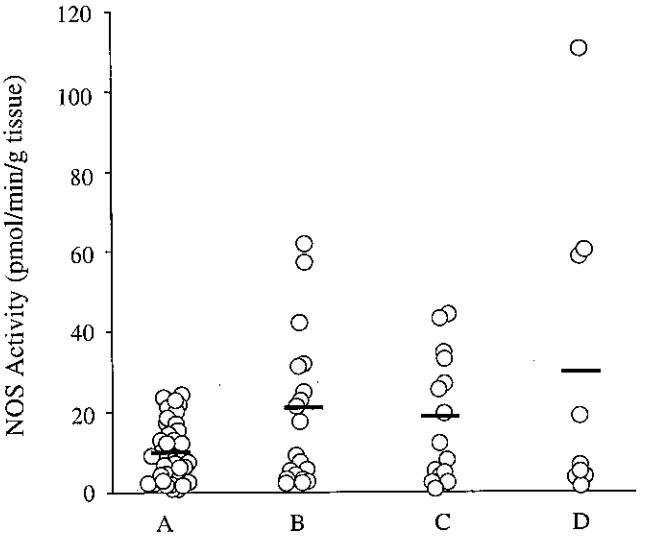

Fig. 3. Total NOS activity of adenocarcinoma classified by grade of malignancy. Grades of malignancy (well, moderately, and poorly differentiated) were assigned on the basis of histopathologic examination. No statistically significant association was recognized between grade and NOS activity. A, Control samples; B, well differentiated samples; C, moderately differentiated samples; D, poorly differentiated samples.

determine NOS activity. Blank values were determined in the absence of the added supernatants. Samples (100 μl) were added to 25 U/ml calmodulin, 0.1 m*M* $CaCl_2$, 1.0 m*M* NADPH, 20 m*M* flavin adenine dinucleotide, 20 m*M* flavin mononucleotide, 5.0 m*M* L-[U-^{14}C]arginine and 5.0 m*M* HEPES (total volume: 200 μl) and incubated for 2 h at 37°C. The background activity was determined as the radioactivity in the absence of 1 m*M* NADPH. To determine c-NOS activity, trifluoperazine was added to the assay mixture to give a concentration of 0.1 m*M*. c-NOS activity was calculated by subtracting the activity of trifluoperazine-containing medium from the total NOS activity.

Statistics The results were evaluated statistically by using Welch's *t* test. A *P* value of 0.05 or less was considered to indicate a statistically significant difference.

RESULTS

NOS activity in normal lung samples NOS activity in normal lung samples resected at the time of surgery was measured as described above. Low NOS activities (<25 pmol/min/g tissue) were observed in these tissues and no statistically significant correlation between the patients' age and NOS activity was found (Fig. 1A).

NOS activity in lung cancer samples The characteristics of the tumor samples examined in this study are summarized in Table I. Total NOS activities were measured in all the samples. As with the normal samples, no significant correlation between the patients' age and NOS activity was observed (Fig. 1B). Lung cancer samples were categorized into 3 types: adenocarcinoma, squamous cell carcinoma, and others (small cell, large cell, adenosquamous carcinoma, and carcinoid). Total NOS activities in adenocarcinoma samples were significantly higher than those in other types of cancers and normal lung samples ($P<0.05$) (Fig. 2). NOS activities in squamous cell carcinoma samples were not significantly different from those in others and normal samples (Fig. 2). Addition of trifluoperazine, a specific inhibitor of calmodulin, to the assay system almost completely suppressed NOS activity in all the samples examined except for 3 adenocarcinoma and 3 squamous cell carcinoma samples. Analysis of adenocarcinoma samples by tumor grade revealed no significant difference in NOS activity (Fig. 3). TNM classification showed that, although T stage did not correlate with NOS activity (Fig. 4), cancer tissues from patients with N2 disease tended to have lower activity than those from patients with N0 and N1 disease (Fig. 5).

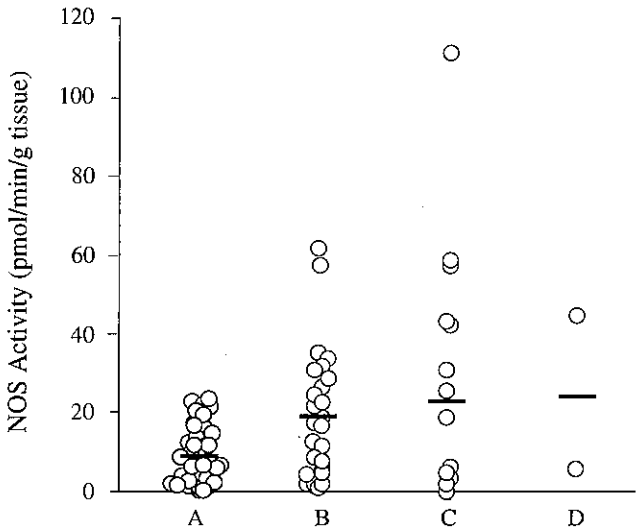

Fig. 4. T stage and total NOS activity in adenocarcinoma samples. Samples were divided into 3 groups according to the T stage. No statistically significant association was observed between T stage and NOS activity. A, Control samples; B, T1; C, T2; D, T3.

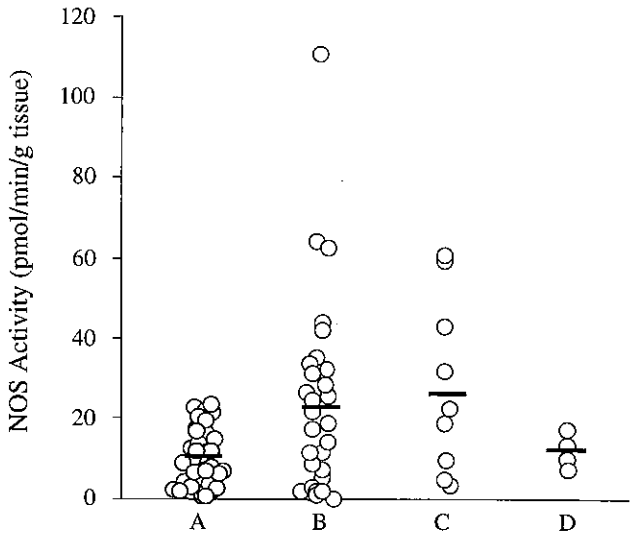

Fig. 5. Lymph node metastasis (N stage) and total NOS activity in adenocarcinoma samples. No statistically significant association was seen between N stage and NOS activity. A, Control samples; B, N0; C, N1; D, N2.

Immunohistochemical studies of tissue sections We performed immunohistochemical analysis to examine the expression pattern of NOS isoforms in tumor and normal samples. The presence of NOS was detected in both high (>40 pmol/min/g tissue) and low NOS activity (<20 pmol/min/g tissue) samples using anti-NOS antibodies as described above (Table II). Although anti-i-NOS and anti-ec-NOS immunoreactivities were essentially absent in normal lung epithelial cells, slight anti-b-NOS immunoreactivity was seen in those tissues (data not shown). All of the high NOS activity tumor samples showed anti-b-NOS immunoreactivity: 5 samples showed a mosaic pattern (Fig. 6, A and B) and 2, a diffuse pattern (data not shown). In the low NOS activity samples, low b-NOS immunoreactivity was seen in 5 adenocarcinoma and 3 squamous cell carcinoma samples (Table II). Fig. 6, C and D present typical patterns of the staining in adenocarcinoma and squamous cell carcinoma, respectively. Immunoreactivity for b-NOS was predominantly found in the cytoplasm of the tumor cells (Fig. 6, A, B, and C). In some of the tumor cells, immunoreactivity also seemed to be present in the nuclei (Fig. 6D). The cells which seemed to show the immunoreactivity in the nuclei were often characterized by a high nuclear-to-cytoplasmic ratio and prominent nucleoli. In 2 adenocarcinoma samples, intense or moderate immunoreactivity for ec-NOS was detected (Fig. 7, A and B). i-NOS immunoreactivity was recognized in 5 adenocarcinoma samples (Fig. 7, C and D). Three squamous cell carcinoma samples with elevated i-NOS activity also showed i-NOS immunoreactivity in tumor cells (Fig. 7E). In one adenocarcinoma sample showing high i-NOS activity, the presence of a significant number of macrophages and lymphocytes that had migrated to the tumor tissues was confirmed by the immunoreactivity to EBM-11 antibody, L26, and UCHL-1 antibody. EBM-11 immunoreactivity was predominantly found in the stroma, where i-NOS immunoreactivity was weakly observed (data not shown).

DISCUSSION

We have demonstrated in this paper that high levels of NOS activity are present in some types of lung cancer, especially adenocarcinoma. The presence of NOS in these tissues was confirmed by immunohistochemistry using monoclonal anti-b-NOS, anti-ec-NOS and anti-i-NOS antibodies. This immunoreactivity was predominantly localized in the tumor cells. It has been well documented that all forms of NOS are expressed in normal human lung epithelial cells and in cell lines derived from lung adenocarcinoma.[24, 25] In fact, all of the tumor samples examined in our study showed immunoreactivity for more than one NOS antibody (Table II). Even in the normal lung tissues, low NOS activity was observed, while some lung cancer tissues showed elevated NOS activity. Immunohistochemical analysis revealed that, although anti-i-NOS and anti-ec-NOS immunoreactivities were not observed in normal lung epithelial cells, slight anti-b-NOS immunoreactivity was seen in those tissues.

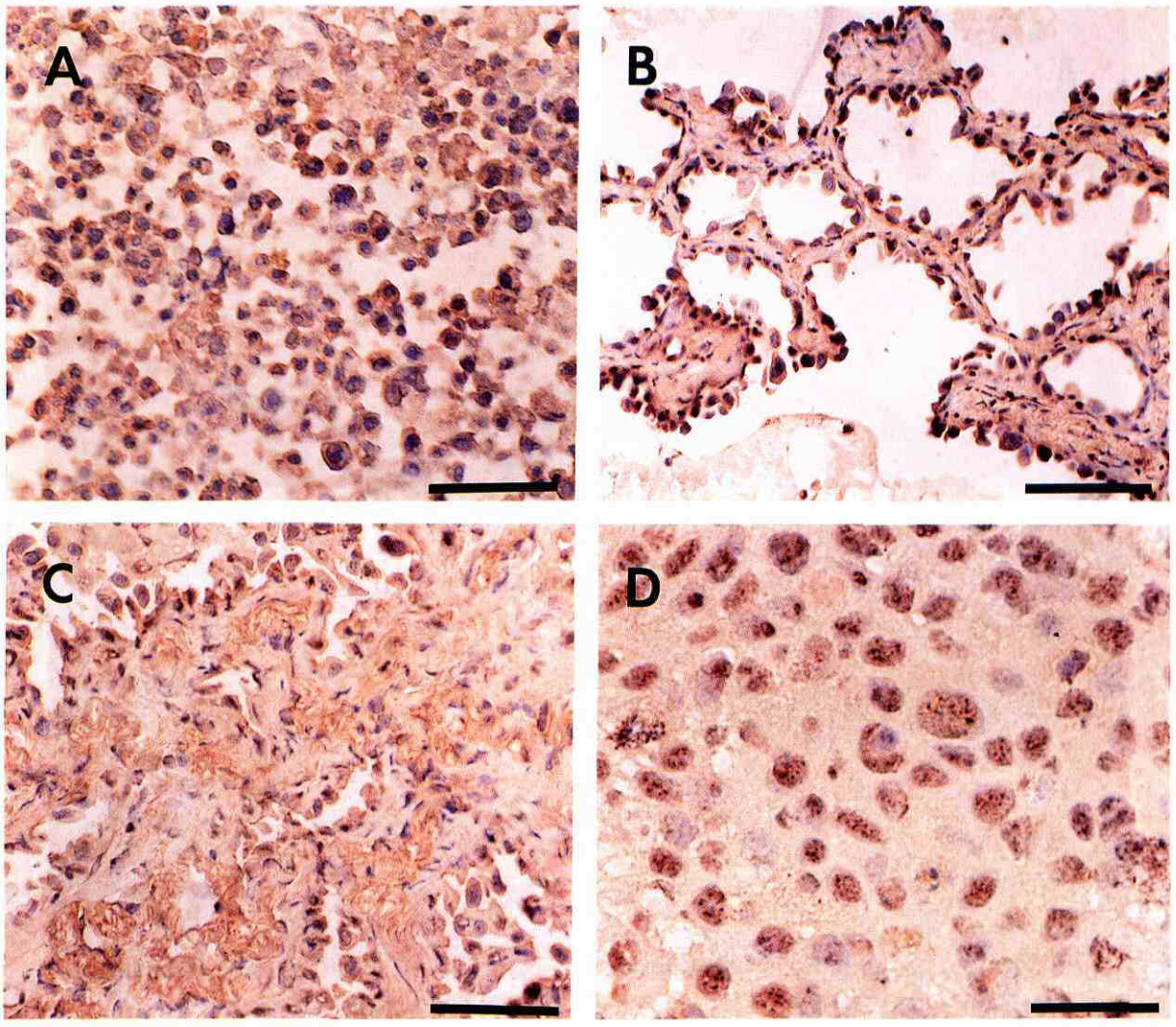

Fig. 6. Immunoreactivity for b-NOS. Immunoreactivity to anti b-NOS antibody was determined as described in the text. A, Mosaic, intense immunoreactivity for b-NOS was seen in this poorly differentiated adenocarcinoma (scale bar, 60 μm). B, Mosaic, intense immunoreactivity for b-NOS was seen in this well differentiated adenocarcinoma (scale bar, 100 μm). C, Mosaic, light immunoreactivity for b-NOS was seen in this moderately differentiated adenocarcinoma (scale bar, 80 μm). D, Diffuse, moderate immunoreactivity for b-NOS was seen in this poorly differentiated squamous cell carcinoma (scale bar, 40 μm). A and B show tissue with over 40 pmol/min/g of NOS activity. C and D show tissue with less than 20 pmol/min/g of NOS activity.

However, the intensity of anti-b-NOS immunoreactivity in tumor samples was much greater than that in normal tissues. Although b-NOS immunoreactivity was mainly observed in the cytoplasm of the cancer cells, the nuclei also seemed to be stained in several cancer samples, suggesting that localization of b-NOS may vary in some types of lung cancer cells, or immunoreactivity found in the cytoplasm may overlap with the nuclei of cancer cells. These results suggest that NOS, especially b-NOS, may play some role in the pathophysiology of lung cancer.

A trifluoperazine inhibition assay revealed that most of the elevation in NOS activity in adenocarcinoma tissues was due to c-NOS, while 3 of 44 adenocarcinoma and 3 of 18 squamous cell carcinoma samples contained predominantly i-NOS. In one cancer sample, a significant number of macrophages had migrated to the tumor tissues and expressed slight immunoreactivity of i-NOS, in

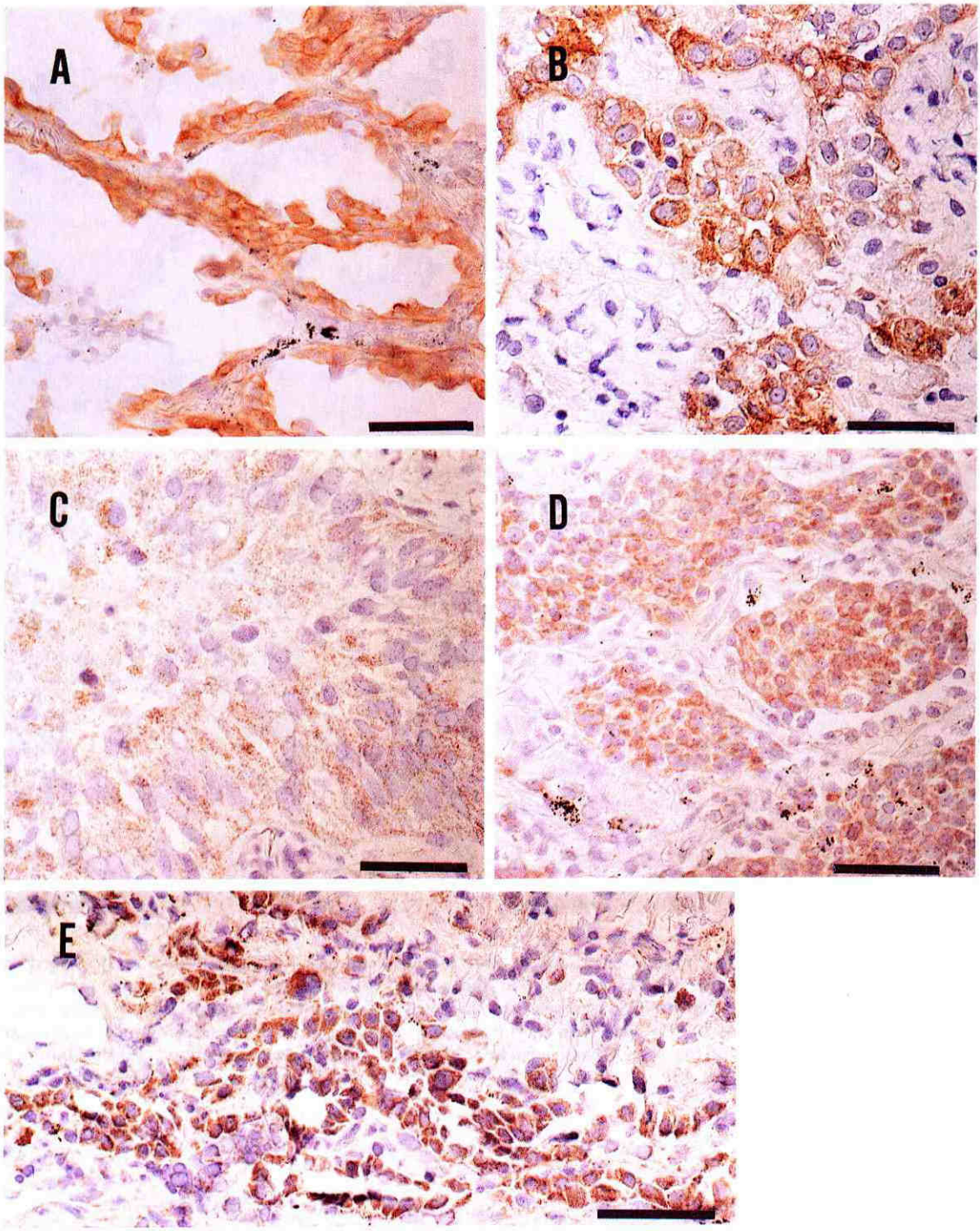

Fig. 7. Immunoreactivity for ec-NOS and i-NOS. A, Diffuse, moderate immunoreactivity for ec-NOS was seen in this well differentiated adenocarcinoma (scale bar, 75 μm). B, Mosaic, intense immunoreactivity for ec-NOS was seen in this moderately differentiated adenocarcinoma (scale bar, 60 μm). C, Diffuse, light immunoreactivity for i-NOS was seen in this poorly differentiated adenocarcinoma (scale bar, 50 μm). D, Diffuse, moderate immunoreactivity for i-NOS was seen in this moderately differentiated adenocarcinoma (scale bar, 60 μm). E, Diffuse, moderate immunoreactivity for i-NOS was seen in this moderately differentiated squamous cell carcinoma (scale bar, 60 μm). B and C show tissue with over 40 pmol/min/g of NOS activity. A, D, and E show tissue with less than 20 pmol/min/g of NOS activity.

addition to the intense immunoreactivity of the tumor cells. EBM-11 antibody, which can detect macrophages, predominantly reacted in the stroma where the slight i-NOS immunoreactivity was observed. These results suggest that i-NOS activity of macrophages did not significantly contribute to the activity in the tumor tissue, and indicate that some types of lung cancer cells predominantly express i-NOS rather than b-NOS and ec-NOS.

NOS activity in tumor tissue did not correlate with the tumor grade of lung cancer in our study, although it has recently been reported that NOS activity correlated with tumor grade in other types of solid tumor.[13–15] In our study, adenocarcinoma samples were classified as having low or high NOS activity. Adenocarcinoma cells containing high levels of NOS may possess some novel biologic characteristics, although we could not confirm this by classification of lung cancer. Histologic and cytologic heterogeneity is characteristic of lung adenocarcinoma, and many cytologic subtypes of adenocarcinoma have been proposed, so cytologic subtypes of adenocarcinoma may correlate with NOS activity. A new classification based on NOS activity of cancer tissues may represent a new prognostic marker for lung adenocarcinoma.

Lung cancer metastasis involves a series of interrelated steps that include motility, invasion, survival in the circulation, adhesion, extravasation, and growth.[26–28] NO produces multiple effects that can influence the outcome of metastasis.[29] These effects include NO-regulated vasodilatation,[30, 31] promotion of angiogenesis[32] and platelet aggregation,[33, 34] which affects tumor cell arrest in capillaries and thus metastasis.[26, 35] Analysis by TNM classification indicated that lymph node metastasis seems to be inversely correlated with NOS activity, suggesting that NO may play a role in the suppression of lymph node metastasis. Jenkins *et al.* proposed that NO may have both pro- and anti-tumor actions depending on its local concentration.[20] Our findings are also consistent with the idea that NO may play a dual role. However, the role of NO in solid tumor biology is still not fully understood, and further investigation of its role in lung cancer is needed.

In summary, NO may play important role(s) in the metabolism and behavior of lung cancer, especially adenocarcinoma. Research in this area may have important diagnostic and therapeutic implications.

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