Nitric Oxide Synthase, Cyclooxygenase 2, and Vascular Endothelial Growth Factor in the Angiogenesis of Non-Small Cell Lung Carcinoma

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ABSTRACT

We have investigated the hypothesis that nitric oxide synthase (NOS2), cyclooxygenase-2 (COX2), and vascular endothelial growth factor (VEGF) protein levels individually demonstrate a direct correlation with microvessel density (MVD) and clinical outcome in human non-small cell lung cancer (NSCLC). Furthermore, we hypothesized that MVD may explain the propensity of certain histological lung cancer subtypes for early metastasis via a hematological route. Immunohistochemically, we studied the protein expression levels of NOS2, COX2, and VEGF and MVD by counting CD31-reactive blood vessels (BVs) in 106 surgically resected NSCLC specimens. NOS2, COX2, and VEGF immunoreactivity were observed in 48, 48, and 58%, respectively, of the study subjects, and their levels correlated with MVD at the tumor-stromal interphase ($P \leq 0.001$). More adenocarcinomas and large cell carcinomas displayed overexpression of NOS2 when compared with squamous cell carcinoma (SCC; $r = 0.44$; $P < 0.001$). NOS2 and COX2 levels were found to correlate positively with VEGF status ($r = 0.44$; $P < 0.001$, 0.01, and 0.03, respectively). These results attest to the significant interaction of these factors in the angiogenesis of NSCLC. Although neither angiogenic factors nor MVD correlated with patient survival, the latter correlated with tumor clinical stage in both squamous (SCC; 73 BVs/mm^2) and non-SCC (78 BVs/mm^2) tumors. These results indicate that angiogenesis is a complex process that involves multiple factors including NOS2, COX2, and VEGF. Furthermore, the role of angiogenesis in the biology of various histological lung cancer types may be different. The complexity of angiogenesis may explain the modest results observed in anti-angiogenesis therapy that target a single protein.

INTRODUCTION

Angiogenesis is essential for tumor growth *in vivo* (1). Cytokines and growth factors, such as TGF[2]-β, TGF-α, platelet-derived growth factor, basic fibroblast growth factor, and VEGF, are known to promote angiogenesis (2–5). Although the up-regulation of these factors may be related, in part, to the host immune system, oncogenes, *e.g.*, K-*ras* (6) and tumor suppressor genes, *e.g.*, p53 (7, 8), can regulate the expression of these angiogenic proteins.

Angiogenesis is a complex process where several proteins and enzymatic pathways converge. COX2, a catalyst in prostaglandin synthesis from arachidonic acid, contributes to the regulation of angiogenesis by various genes, including platelet-derived growth factor, VEGF, fibroblast growth factor-α, and TGF-β. Using selective inhibitors of COX2, Tsujii *et al.* (9) were able to block the expression of several angiogenic factors including VEGF. Furthermore, Celecoxib (NS398), a specific COX2 inhibitor, has been shown to inhibit the growth of 97% of colon cancer cells by reducing the number of hotspots in the tumor-stromal bed (10).

Another angiogenesis modulator is NOS2, which appears to exert a direct effect on several angiogenic factors such as VEGF. Through the depletion of intracellular iron, the expression of VEGF is activated (11). These observations were further confirmed by Ambs *et al.* (12), who reported higher VEGF protein and mRNA levels in NOS2-expressing cells when compared with the control vector-containing cell lines. These levels were reduced when an inhibitor was added to the culture (12). NOS2 can induce COX2 through the overexpression of nuclear factor-κB and its dimer subunit, p60/p65, which enters the nucleus and induces NOS2 and COX2 among other genes (13). Although VEGF levels appear to be mediated primarily by hypoxia-induced factor-1 (7), there is evidence to suggest that both NOS2 and COX2 may play a role in the signaling pathway that leads to its overexpression. NOS2 is known to function as an up-regulator of VEGF-regulated kinases and mitogen-activated protein kinases (14). In contrast, wild-type p53 exerts a

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[2] The abbreviations used are: TGF, transforming growth factor; VEGF, vascular endothelial growth factor; COX, cyclooxygenase; NOS2, nitric oxide synthase; NSCLC, non-small cell lung cancer; MVD, microvessel density; ADC, adenocarcinoma; LCC, large cell carcinoma; BV, blood vessel; MAb, monoclonal antibody.

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Table 1 NOS2, COX2, VEGF, and MDV status in relation to patients' demographic data, cancer histopathology, and clinical staging

	No.	NOS2 (%)	COX2 (%)	VEGF (%)	MVD/m^2
All	106	51 (48)	51 (48)	61 (58)	76.3
Male	62	30 (48)	27 (44)	36 (58)	74.6
Female	44	21 (48)	24 (55)	25 (57)	78.7
Smoking history					
Smokers	102	49 (47)	50 (48)	55 (54)	76.5
Nonsmokers	4	1 (25)	3 (75)	2 (50)	71.0
Histology					
ADC	55	28 (49)	29 (53)	34 (56)	79.0
SCC	29	7 (27)	14 (48)	13 (45)	64.0
LCC	22	16 (73)	11 (50)	14 (64)	89.0
Tumor size					
T_1	24	17 (71)	11 (46)	18 (75)	78.3
T_2	43	30 (70)	22 (51)	28 (65)	82.1
T_3	39	21 (54)	24 (62)	25 (64)	67.5
Lymph node status					
N_0	56	36 (64)	27 (48)	35 (63)	72.4
N_1	30	19 (63)	19 (63)	21 (70)	76.9
N_2	18	12 (67)	11 (61)	13 (72)	83.0
Clinical stage					
I	52	35 (67)	29 (56)	35 (67)	72.1
II	19	11 (58)	8 (42)	13 (68)	80.4
III	26	17 (65)	16 (62)	16 (62)	79.2
IV	8	5 (63)	6 (75)	7 (88)	86.9

significant influence on the process through the induction of thrombospondin-1 (15), which down-regulates NOS2, COX2, and VEGF directly (15), or though the activation of pro-TGF-β to active TGF-β, a major suppressor of these protein levels (16). In addition, p53 *trans*-suppresses COX2 levels directly (17). Our previous work has shown that wild-type p53 may play a more central role in the regulation of angiogenesis through its direct control loop of NOS2 and VEGF (8, 12, 18).

Tumor angiogenesis has received attention as a plausible candidate in relation to prognosis in NSCLC. Several studies have found that VEGF, VEGF receptors, and MVD have a direct correlation with prognosis (2, 19), node-free intervals, and relapse- and recurrence-free periods (20). However, other studies were not as conclusive (21, 22). The discrepancy in these results can be attributed to several variables, such as tumor histology and the type of vascular marker used to measure MVD.

In a study of 108 NSCLC samples, where tissues were stained with CD34 and factor VIII, Yano *et al.* (19) concluded that MVD, as measured by CD34 and VEGF levels, correlated with survival, postoperative recurrence, and metastasis in ADC. No such correlation was found when MVD was measured by factor VIII (23). Giatromanolaki *et al.* (24) found a positive correlation with CD31 MVD but not with factor VIII. In a separate study of 87 NSCLC samples, Shijubo *et al.* (25) reported that the levels of VEGF, osteopontin, and MVD were higher and correlated with a poor clinical outcome in ADC patients in comparison with SCC. The discrepancy as to the role of angiogenesis and patient survival may be related to the morphology of BVs and the type of VEGF isoform present. Data from a study of 500 NSCLC samples showed that neoangiogenesis could differ in its morphological appearance. Three patterns were characterized by the destruction of lung parenchyma and the production of new BVs. The fourth pattern, which was called alveolar and presented in 16% of the tumors, showed a lack of parenchymal destruction and the absence of both tumor-associated stroma invasion and neoangiogenesis (26). Furthermore, VEGF forms four isoforms involving alternative splicing, resulting in amino acid sequences ranging from 121 to 206. Isoform expressions were measured on normal and transformed lung and colon tissues. Shorter peptides (121 and 165) were found at higher levels in malignant tissues, whereas longer isoforms were observed in normal tissues. These observations suggest that during malignant transformation, a switch takes place to a more active bioavailable and diffusible form of VEGF through alternative splicing (27).

We have studied the levels of NOS2, COX2, and VEGF protein levels in 106 surgically resected NSCLC tumors and correlated these levels with MVD. Furthermore, we investigated the effect of these markers on tumor size, histology, and clinical outcome.

MATERIALS AND METHODS

Patient Population. One hundred and six patients with surgically resected NSCLC tumors were included in this study, all of which were collected prospectively from the Mayo Clinic (Rochester, MN) from 1992 to 1993. The epidemiological data including demographics, family history, occupational exposure, medical history, and histopathological data on these patients have been reported previously (28). The patients included 62 males and 44 females. All but 4 patients were cigarette smokers, with an average pack-year history of 63.1 for men and 41.5 for women. Histologically, 55 cases (52%) had ADCs, 29 had SCCs (27%), and 22 had LCCs (21%). The clinical stages are summarized in Table 1. The tumor status (T) was available on all patients. In addition, the lymph node status and the clinical stage were known for all but 2 and 1 patient, respectively.

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Immunohistochemical Methods. In addition to H&E light microscopy examination, 4-μm tissue sections were cut from paraffin blocks and mounted on electrically charged glass slides. The sections were heated in an oven at 60°C for 45 min, deparaffinized in three changes of xylene solution, and dehydrated in decreasing alcohol grades for 5 min each. Endogenous peroxidase was quenched by immersion in 3% hydrogen peroxide for 30 min. An antigen retrieval method followed, using a microwave at 140 joules and antigen retrieval solution (BioGenex, San Ramon, CA) for 30-min periods. The sections were incubated overnight at 4°C in moisture chambers with a battery of MAbs including anti-NOS2, anti-COX2 (Transduction Laboratories, Lexington, KY), and anti-VEGF and anti-CD31 (Dako Corp., Santa Barbara, CA) at dilutions of 1:125, 1:25, 1:50, and 1:50, respectively. Antibody binding was detected by subsequent incubation with a biotinylated secondary antibody and streptavidin peroxidase complex (ABC kit; Vector Labs, Burlingame, CA). Chromogenic development was obtained by the immersion of sections in a 3,3′-diaminobenzidine solution (0.25 mg/ml with 3% hydrogen peroxide). The slides were counterstained with Mayer's hematoxylin (Biogenex) and coverslipped after the application of mounting medium. The results for NOS2, COX2, and VEGF MAbs were reported as a combined score of distribution and intensity as described in previous work on p53 (28). For the purpose of statistical analysis for anti-NOS2, anti-COX2, and anti-VEGF, a score of 0 or 1 was considered baseline or normal expression, whereas scores of 2 or more were considered overexpression. The anti-CD31 results were reported as numbers of BVs <20 μm in diameter. For each section, the number of BVs in the so-called "hotspots" at the tumor-stromal interphase (1) were captured at $\times 10$ in five fields, using Spot software program version 2.1 (Diagnostic Instruments, Inc., Sterling Heights, MI), and counted manually. Then the average of the BV count/mm samples was calculated (1).

Statistical Analysis. Statistical analysis was performed using SPSS version 10 (Chicago, IL). The Spearman rank-order correlation coefficient was used to assess the relation among VEGF, NOS2, COX2 (using the combined score of intensity and distribution), MVD, and other continuous variables. Associations among a variety of variables, including, gender, tumor histology, smoking history, and family history of malignancy, were evaluated using the χ^2 test for heterogeneity or Fisher's exact test as appropriate. Kaplan-Meier analysis was used to assess the relation of disease-free survival to overexpression using the log-rank test. Student's *t* test was used to compare the continuous variables including age at diagnosis and lifetime smoking dose expressed in pack-years, by the nominally classified VEGF, NOS2, and COX2. Associations were considered statistically significant if the two-tailed *P* was <0.05.

RESULTS

The status of NOS2, COX2, VEGF, and MVD are summarized in relation to demographic, histopathological, and clinical data in Table 1. Of 106 cases, 51 tumors showed anti-NOS2 immunoreactivity of 2 or higher (48%; Fig. 1*A*). Fifty-one (48%) samples demonstrated a score of 2 or higher for anti-COX2 (Fig. 1*B*), and 61 tumors (58%) showed anti-VEGF scores of 2 or higher (Fig. 1*C*). Both NOS2 and COX2 levels showed a positive correlation with VEGF overexpression ($P < 0.001$ and $P < 0.03$, respectively). NOS2 levels of overexpression were higher in certain histological subtypes, such as LCCs (16 of 22; 73%) and ADCs (28 of 55; 51%). NOS2 overexpression in SCC was uncommon and only seen in 7 of 29 samples (24%; $P < 0.002$). However, increased expression of COX2 or VEGF did not show a preference to any histological subtypes of tumor. Increased levels of VEGF were seen in 34 ADCs (62%), 13 SCCs (45%), and 14 LCCs (64%). COX2 overexpression was present in 26 ADCs (47%), 14 SCCs (52%), and 11 LCCs (50%). In addition, elevated VEGF levels were found in 39 (76%) and 36 (71%) of all tumors.

MVD showed a mean of 78 BVs/mm^2 in non-SCCs (ADCs and LCCs combined) as compared with SCCs with a mean of 73 BVs/mm^2 (Fig. 1, *D* and *E*). Furthermore, only 20% of SCCs had MVD of >100 BVs/mm^2 when compared with 30% of non-SCC tumors. Individual levels of VEGF, COX2, and NOS2 were all significantly correlated with MVD directly with *r* values at 0.46, 0.32, and 0.47, respectively ($P \leq 0.001$). Although the tumor size, lymph node status, patient outcome, and survival did not correlate with NOS2, COX2, VEGF overexpression or MVD ($P > 0.05$). Overall, the mean MVDs in clinical stages I–IV were not statistically different (72, 80, 79, and 87/mm^2; 56% of the tumors with <100 BVs/mm^2 were in clinical stage I when compared with 38% of the tumors with >100 BVs/mm^2). In addition, 44% of the tumors with <100 BVs/mm^2 were in stages II–V *versus* 62% of the tumors with MVD >100 BVs/mm^2. When tumors were segregated histologically, MVD showed a more significant correlation with non-SCC clinical stage than SCC tumors ($P < 0.0001$). Furthermore, the NOS2 and COX2 levels did not correlate with patient pack-years of tobacco smoking or age at diagnosis, or the gender of the patients ($P > 0.05$).

DISCUSSION

The data presented here highlights three significant findings: (*a*) NOS2, COX2, and VEGF exhibit overexpression in ~50% or more of the tumors; (*b*) increased levels of NOS2, COX2, and VEGF are directly correlated with MVD, as measured by the number of CD31-reactive BVs at the tumor-stromal interphase; and (*c*) the incidence of NOS2 overexpression was more common in ADC and LCC tumors when compared with SCCs. Although the NOS2 and COX2 status have been investigated in several neoplastic and preneoplastic conditions, little work has been done on the lung. In a separate work and on a limited number of samples, Ambs *et al.* (8) were able to detect increased levels of NOS2 in NSCLC samples. Furthermore, Fujimoto *et al.* (29) in a study of 72 primary NSCLC samples were able to detect high levels of NOS2 more frequently in ADCs than other histotypes. In five of eight cases with high NOS2 overexpression, a p53 transversion, G:C to T:A, was identified, suggesting that NOS2 may play a key role in the carcinogenesis of certain histological types of lung cancer (30). Although our data showed increased overexpression of NOS2 in ADC and LCC histological subtypes, we were not able to

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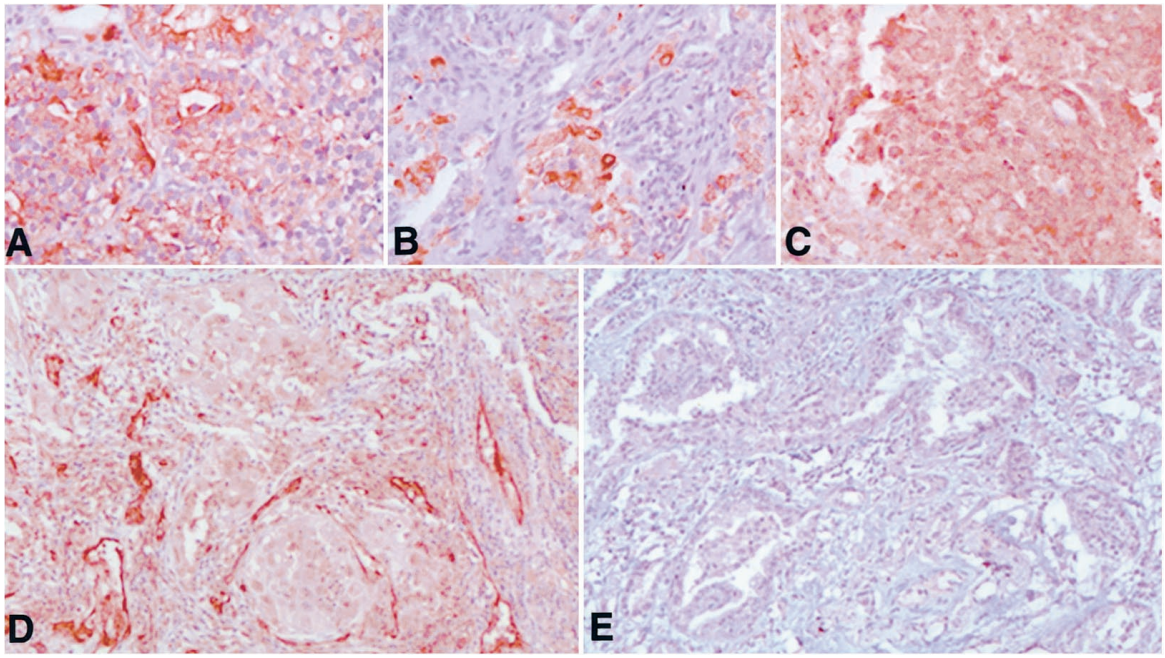

Fig. 1 Angiogenic proteins in a NSCLC case. *A,* NOS2 expression in a female patient with ADC. Reactivity is seen in the cytoplasm. This case received a score of 2. *B,* COX2 status. In addition to tumor cell reactivity with anti-COX2 MAb, adjacent mononuclear inflammatory cells show the enzyme expression as well. *C,* represents the VEGF status. Intense cytoplasmic staining is observed in tumor cells. Reactivity also is present in intratumoral blood vessels. ×200. CD31 immunoreactivity in two samples with different BV density. High BV count is seen in *D* as compared with low vascularity in *E*. ×100.

correlate with p53 G→T transversions that were published earlier by our group (28). On the other hand, the ADC subtype presents with several unique features both clinically and biologically. The tendency to predominate in women, and individuals with no known history of tobacco exposure (31), and the tendency for early hematological dissemination suggests a different carcinogenic pathway, perhaps involving endocrine factors (32). Our work and others have shown a difference in frequency and spectra of p53 mutations in various NSCLC histological subtypes. Overall, p53 mutations were 2.5 higher in SCCs with greater propensity for G→T transversions than those observed in ADCs (33, 34). The MVD in our cohorts of tumors may explain the higher incidence of early metastasis observed in the course of ADCs, because the mean MVD in ADCs was 79/mm^2, compared with SCCs, with a mean of 64 ($P < 0.05$).

Our angiogenesis data did not correlate with patients' outcome and prognosis. This is not surprising in light of contradicting data on the role of angiogenesis, because biological and prognostic markers have been reported (19, 22, 25). These conflicting results may be attributed to several variables. In a study of 108 NSCLC samples, Yano *et al.* (19) showed that MVD levels as measured by CD34 had a direct relation with survival; however, using the same tumor samples, similar results could not be observed with factor VIII. Similarly, CD31, the most widely used vascular marker, is known to recognize an epitope present in both mature and immature venules and capillaries. Recently, Kakolyris *et al.* (35) observed that the presence of immature newly formed BVs can serve as a better indicator for neoangiogenesis. However, more tumors with MVD of >100 BVs/mm^2 presented in higher clinical stages than those with MVD of <100 BVs/mm^2 (62% *versus* 44% with a $P < 0.01$). Furthermore, the results were more significant when tumors were separated based on their histologies ($P < 0.001$).

The variability of the results may be attributed to the histological type of lung cancer used in these studies. Some investigators have reported that MVD and VEGF and its receptor status are associated with ADCs (19, 21) but not SCCs (34), whereas others have suggested only SCC tumors showed such correlation (36), and occasionally with a subgroup of SCC tumors (37). The methodology to measure MVD (manual *versus* computer-assisted imaging) as well as the site where MVD is measured can contribute to the discrepancy of the role of angiogenesis and patients' survival. Some investigators have used the number of BVs per high-power field as an index for MVD (38), whereas others have used the number of hotspots at the tumor-stromal interphase (2). Still, other factors for this controversy may be attributed to the epitope of anti-VEGF MAb used. Evidence of several different VEGF isoforms secreted by different NSCLC tumors has been reported (35). The morphology of the new vessel formation may yet represent another variable factor, because only BVs that display host-stromal infiltration appear to correlate with poor outcome (27). Finally, our cohort represented lung cancer patients that were good candidates for surgical resection. Those with T4 tumors or N3 were not included in this group. This clinical bias also may explain the lack of correlation of MVD and other angiogenic factors studied with patient survival or tumor behavior.

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Angiogenesis appears to play a significant role in cancer progression and evolution. Thus, novel therapies have emerged that use this phenomenon as a target for cancer therapies. To date, more than three dozen clinical trials have been approved that target tumor angiogenic and antiangiogenic factors such as endostatin, VEGF, IFN-α2a, and α-2-macroglobulin, or tissue inhibitors of metalloproteases and pharmaceutically prepared agents such as selenium-based drugs, *e.g.,* Thalidomide, Aplidine, and others (39–42). However, we have shown that COX2, NOS2, and VEGF levels correlate with the degree of MVD individually, and their levels appear to correlate with each other. Thus, it is likely that angiogenesis-based treatment protocols that target individual proteins will have modest yields and perhaps disappointing results.

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