Genetic Ablation of Inducible Nitric Oxide Synthase Decreases Mouse Lung Tumorigenesis

Lori R. Kisley, Bradley S. Barrett, Alison K. Bauer, Lori D. Dwyer-Nield, Benjamin Barthel, Amy M. Meyer, David C. Thompson, and Alvin M. Malkinson

Departments of Pharmaceutical Sciences [L. R. K., B. S. B., L. D. D-N., B. B., D. C. T., A. M. M.] and Pharmacology [A. M. M.], University of Colorado Cancer Center, University of Colorado Health Sciences Center, Denver Colorado 80262, and Laboratory of Pulmonary Pathology, National Institute of Environmental Health Sciences, NIH, North Carolina 27709 [A. K. B.]

ABSTRACT

Inducible nitric oxide synthase (iNOS) content is elevated in human lung adenocarcinomas, and lung cancer patients exhale more nitric oxide (NO) than healthy individuals. The mechanism of this association of chronically elevated NO with tumorigenesis has not been defined. We investigated the role of iNOS in murine lung tumorigenesis, a model of human lung adenocarcinoma, using wild-type (+/+) and iNOS (−/−) mice. Genetic disruption of iNOS decreased urethane-induced lung tumor multiplicity by 80% (P < 0.0001). iNOS protein was expressed in lung tumors growing in wild-type mice and bronchiolar Clara cells isolated from normal mouse lungs, but was undetectable in whole lung extracts by immunoblotting. Because NO regulates vascular endothelial growth factor (VEGF) expression in other systems, we examined the effect of iNOS deficiency on VEGF protein concentration in mouse lung tumors. VEGF concentration was 54% lower in tumors isolated from iNOS (−/−) mice versus controls, implying that NO modulates angiogenesis in these tumors. Lung tumors also have elevated levels of cyclooxygenase (COX).-1 and COX-2 contents relative to normal lungs, but iNOS deficiency did not change COX expression in the tumors. Chronic inflammation predisposes mice to lung tumorigenesis; accordingly, we examined whether butylated hydroxytoluene-induced chronic lung inflammation was influenced by iNOS deficiency. Butylated hydroxytoluene-induced alveolar macrophage infiltration was unaffected by iNOS (−/−) status, suggesting that although NO is a critical mediator of mouse lung tumorigenesis, it is not essential in this model of lung inflammation. The substantial (80%) reduction in lung tumor multiplicity in iNOS (−/−) mice strongly supports examining iNOS-specific inhibitors as potential lung cancer chemopreventive agents.

INTRODUCTION

Lung cancer is the leading cause of cancer-related deaths in the United States (1). The prevalence of non-small cell lung carcinoma, including its most common subtype, AC, is increasing in both smokers and nonsmokers (2). In general, AC has metastasized before clinical symptoms become apparent, which reduces successful treatment options (3). Consequently, the most effective means to combat AC has metastasized before clinical symptoms become apparent, which reduces successful treatment options (3). Consequently, the most effective means to combat AC is through chemoprevention aimed at relevant molecular targets (4). The ubiquitous signaling molecule, NO, is synthesized by the NOS enzymes; iNOS is the primary NOS responsible for heightened NO production during inflammation and various pathologies.

The absence of iNOS (5) or inhibition of its activity (6, 7) attenuates rodent colon cancer development. Because iNOS influences physiological and pathological processes that mediate tumorigenesis, such as inflammation (8, 9) and angiogenesis (10), we postulate that iNOS may be a critical regulator of tumor development in other organs as well, including the lungs.

Increased levels of exhaled NO are detectable in lung cancer patients, and iNOS content is elevated in lung tumor cells, alveolar and tumor-associated macrophages, pulmonary endothelium, and airway epithelium in these patients (11). NO activity and expression are higher in human lung AC than in other forms of lung cancer or normal lung (12). NO has been linked to early events in the tumorigenic process, including DNA damage, lipid peroxidation, and regulation of inflammation (13, 14), all of which can encourage neoplastic growth. NO may also modulate later stages of tumorigenesis, including neoangiogenesis and metastasis (15, 16). For example, NO induces VEGF expression in vascular endothelium, vascular smooth muscle, and tumor cells (17–19). Rat alveolar macrophages exhibit higher iNOS expression in areas of pulmonary inflammation (20), and elevation of the alveolar macrophage population enhances murine lung tumorigenesis (21, 22). A role for iNOS and its catalytic product, NO, has not been established for murine lung AC, a model that closely resembles human AC in its histological and molecular features (23, 24).

We examined the induction of lung tumorigenesis in wild-type (+/+) and iNOS (−/−) mice, and measured VEGF protein content in tumors from these mice to evaluate the contribution of iNOS to lung angiongenesis. iNOS-deficient mice developed fewer lung tumors than wild-type mice, and the VEGF content of these tumors was reduced compared with wild-type mice. iNOS and VEGF protein increased in tumors relative to the surrounding tissue in wild-type mice. We hypothesize that the dramatic inhibition of lung tumorigenesis by iNOS deficiency is because of the role of NO in angiogenesis. Elevated NO because of chronic inflammation may encourage carcinogenesis (13); chronic pulmonary inflammation predisposes to lung tumorigenesis according to both epidemiological studies (25) and experimental oncogenesis (21, 22, 26). We demonstrated previously elevated pulmonary COX enzyme content during mouse lung chronic inflammation (21) and tumorigenesis (27). In certain extrapulmonary in vitro and in vivo systems, iNOS and COX enzymes and their catalytic products modulate each other’s activity to regulate inflammation (28–30). Therefore, we examined COX expression in lung tumors from iNOS (+/+) and (−/−) mice, but found no evidence to support a relationship between NO and COX enzyme expression or activity in mouse lungs.

MATERIALS AND METHODS

**Animals.** Male B6/129P-Nos2tm1Lau (iNOS KO, −/−), B6/129P-F2 (wild-type, iNOS +/+) and A/J mice, 5–6 weeks of age, were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in the University of Colorado Center for Laboratory Animal Care. The properties of these mutant mice have been described; they are normal in appearance, growth rate, reproduction, and histology, but peritoneal macrophages isolated from these mice are inca-
pable of generating NO when stimulated with LPS (31). Mice were provided with a standard antioxidant-free laboratory chow (Agway Prolab 3000; Agway, Inc., Syracuse, NY) and water ad libitum, maintained on hardwood bedding under a 12-h light/dark cycle, and weighed weekly for the duration of the experiments.

Urethane Carcinogenesis. Wild-type and iNOS KO mice were injected i.p. with 1 g/kg urethane (Sigma, St. Louis, MO) once weekly for 7 consecutive weeks, and tumors were enumerated and sized using a digimatic caliper (Mitutoyo, Tokyo, Japan) 16 weeks after the initial urethane injection (32). A subset of mice (n = 3) was perfused with 0.9% saline through the right pulmonary artery, the lungs inflated with 10% (v/v) formalin, embedded in paraffin, and 4 μm sections taken for immunohistochemical analysis. Protein extracts prepared from dissected, homogenized tumors from the remaining mice in each treatment group were used for Western blotting analysis. As iNOS KO mice had few tumors per mouse, tumors from 3–4 mice were pooled to provide adequate amounts of protein for Western blotting. A/0 mice were administered a single i.p. injection of 1 g/kg urethane, and tumors were isolated for protein extraction 7 months after the injection. Normal-appearing lung tissue from tumor-bearing mice (uninvolved tissue) and age-matched, saline-injected mice were used as controls.

Clara Cell Isolation (33). Normal lungs from A/0 mice were digested with elastase (Worthington Biochemical, Freehold, NJ) and detached Clara cells separated from macrophages by adherence to an IgG-coated (Sigma) plate. The cells were harvested for protein, disrupted by Dounce homogenization, separated into soluble and particulate fractions by 16,000 × g centrifugation, and used for immunoblotting.

Western Blotting from Lung Extracts. Preparation of extracts from both lungs and lung tumors for Western blotting was performed as described previously (27). Briefly, soluble protein fractions (100 μg of protein per lane) were separated on 10% polyacrylamide gel, blotted, and incubated with a rabbit polyclonal antibody to iNOS (1:500; BD Transduction Laboratories, Lexington, KY) followed by a rabbit IgG secondary antibody (1:10,000; Santa Cruz Biotechnology, Santa Cruz, CA). For analysis of VEGF, soluble protein fractions were separated on a 15% polyacrylamide gel, blotted, and incubated with a rabbit polyclonal antibody to VEGF (A-20; 1:100; Santa Cruz) followed by protein A secondary antibody (1:10,000; BD Transduction). To confirm even protein loading of the gels, the membranes were stained with 0.1% Ponceau S (Fisher Biotech, Fair Lawn, NJ) in 5% acetic acid.

Immunohistochemistry (27). Four-μm sections were deparaffinized and rehydrated using serial ethanol washes. Endogenous peroxidase activity was inhibited by incubating sections with 3% H2O2 in methanol. Antigen retrieval was performed by heating sections in 100 mM citrate buffer (pH 6.0) for 20 min. Sections were blocked with 10% rabbit (COX-1 and COX-2) or 10% goat (iNOS, ecNOS, nNOS, and VEGF) serum (Vector Laboratories, Burlingame, CA). Primary COX-1 and COX-2 (1:2000 dilution; Santa Cruz), iNOS (1:75; BD Transduction), ecNOS (1:100; Santa Cruz), nNOS (1:200; Santa Cruz), or VEGF (1:1000; Santa Cruz) antibodies were added, and sections were incubated overnight at 4°C. Secondary biotin-conjugated rabbit antibody (COX-1 and COX-2) or goat antirabbit (iNOS, ecNOS, nNOS, and VEGF) antibodies was applied, followed by a peroxidase-conjugated streptavidin tertiary antibody complex (Vector); 3,3-diaminobenzidine was used as the peroxidase substrate (Sigma Chemical Co.) for visualization. Sections were counterstained with hematoxylin (Sigma). Specificity of the COX enzymes has been demonstrated previously (27). In our current studies, the absence of iNOS staining in the iNOS (−/−) mice confirms the specificity of the iNOS antibody. The a-20 VEGF antibody recognizes all of the VEGF isoforms. Images were acquired using a Nikon SMZ1500 microscope and Image Pro Plus software (Media Cybernetics, Silver Spring, MD).

BHT Induction of Macrophage Recruitment. Wild-type and iNOS KO mice (n = 10/group) were injected i.p. with 150 mg BHT/kg body weight. This initial BHT dose was followed by once-weekly injections of 200, 300, and 300 mg BHT/kg body weight for a total of four BHT injections. One-hundred μl BHT dissolved in corn oil (Mazola) was injected i.p.; control mice (n = 5/group) received an equivalent volume of vehicle. This BHT dosing schedule induces chronic pulmonary inflammation and promotes lung tumorigenesis (21). Previous studies demonstrated that alveolar macrophage infiltration after chronic BHT dosing is maximal 6 days after the final BHT injection (21). Therefore, 6 days after the final BHT injection, mice were anesthetized, the trachea cannulated, and the lungs lavaged three times with 1 ml of PBS containing 0.6 mM EDTA (21). Inflammatory cell infiltration was determined by pooling lavaged samples from each animal and counting cells using a hemocytometer. Differential cell counts based on cell morphology as determined by a modified Wright’s stain (performed by the University Hospital Clinical Laboratory, Denver, CO) classified the infiltrating cells as macrophages, lymphocytes, neutrophils, or eosinophils.

Statistical Analysis. Data are presented in most graphs as mean ± SE, and analyzed by one-way ANOVA with a Newman-Keuls post hoc test (GraphPad Software, San Diego, CA). A Student’s unpaired t test was used when comparing two experimental groups. Significance was accepted at P < 0.05.

RESULTS

iNOS (−/−) mice developed 80% fewer tumors than wild-type mice (Fig. 1; P < 0.0001). iNOS deficiency did not affect tumor incidence [86% of wild-type and 82% of iNOS (−/−) mice developed at least one pulmonary adenoma]. Tumor size was also unaffected by the lack of iNOS (1.0 ± 0.02 mm in its largest diameter in wild-type mice, 1.0 ± 0.05 mm in iNOS (−/−) mice).

To investigate possible mechanisms to account for the importance of NO in mouse lung tumorigenesis, indicated by this dramatic decrease in tumor multiplicity in iNOS (−/−) mice, we examined pulmonary iNOS expression. iNOS protein is virtually undetectable by immunoblotting in normal mouse lung tissue and in uninvolved tissue (i.e., macroscopically normal-appearing lung tissue adjacent to tumors) collected from tumor-bearing mice. Lung tumors induced by urethane exhibited substantial iNOS expression, as did Clara cells isolated from normal mice, one of the two potential cells of origin for lung tumors (Ref. 34; Fig. 2A). Localization of iNOS in Clara cells was confirmed when normal mouse lung tissue was immunostained for iNOS (Fig. 2B). As described for human and rat lungs (20, 35), iNOS staining was primarily limited to bronchiolar epithelial Clara cells.

In confirmation of the immunoblotting results, iNOS was observed by immunohistochemistry in tumors from wild-type (+/+) mice (Fig. 3A). iNOS staining was apparent throughout the tumor epithelium and particularly intense in cells located at the tumor periphery. As expected, iNOS staining was not observed in tumors from iNOS (−/−) mice (Fig. 3B). To determine whether expression of the constitutive NO synthases, ecNOS and nNOS, was influenced by the iNOS (−/−) genotype, lung tumors from +/+ and iNOS (−/−) mice were stained for ecNOS and nNOS. ecNOS was expressed in lung tumors (Fig. 3, C and D) and in endothelial cells, macrophages, and Clara cells, as has been reported (data not shown; Refs. 35–37) with no apparent differ-

![Fig. 1. Effect of iNOS (−/−) genotype on lung tumorigenesis. iNOS KO mice (n = 17) develop fewer urethane-induced tumors than wild-type (WT) mice (n = 22), *P < 0.0001 by Student’s unpaired t test, compared with wild-type. Each point represents the number of urethane-induced lung tumors in a single mouse; horizontal lines represent means.](https://example.com/fig1.png)
ences between wild-type (+/+) and iNOS (−/−) mice in staining intensity or cellular distribution. Little nNOS immunostaining was observed in lung tumors derived from either +/+ or iNOS (−/−) mice (Fig. 3, E and F), although staining was observed in bronchiolar and alveolar epithelial cells (36, 38, 39). Together, these findings indicate that the absence of iNOS does not stimulate any compensatory up-regulation or cellular redistribution of either ecNOS or nNOS. Enhanced COX-1 and COX-2 immunohistochemical staining in urethane-induced mouse lung tumors has been reported (27). We observed no differences in COX-1 or COX-2 staining in lung tumors induced in +/+ versus iNOS (−/−) mice (Fig. 4, A–D).

To examine any relationship between NO and VEGF in murine lung tumorigenesis, we compared the relative VEGF contents of normal lung tissue, uninvolved tissue from a tumor-bearing mouse, lung tumors in wild-type (+/+) mice, and isolated bronchiolar epithelial Clara cells. VEGF expression was much greater (124% higher) in lung tumors and in isolated Clara cells (150% higher) than in normal lung tissue (Fig. 5A). Localization of VEGF in Clara cells was confirmed by immunohistochemistry on normal mouse lung tissue (Fig. 5B). Intense VEGF staining was present in Clara, endothelial and alveolar type II cells. In tumors derived from iNOS (−/−) mice, VEGF concentrations were 54% lower than in tumors from wild-type (+/+) mice (P < 0.05; Fig. 6). In many iNOS (−/−) tumor samples, VEGF protein was undetectable.

To ascertain NO involvement in the chronic pulmonary inflammation that predisposes mice to lung carcinogenesis (22), we examined macrophage infiltration, that inflammatory measure most directly associated with lung tumorigenesis (22, 26), in +/+ and iNOS (−/−) mice. Chronic BHT treatment doubled macrophage infiltration into BAL fluid in wild-type (+/+) mice (P < 0.05; Fig. 7). iNOS deficiency did not affect this BHT-induced elevation in the number of BAL macrophages. In addition, iNOS deficiency did not influence pulmonary contents of PGE2, or COX-1, COX-2, ecNOS, or nNOS protein contents in untreated mice (data not shown).

DISCUSSION

Human lung epithelial cells constitutively express iNOS (20), and dysregulated NO production may enhance lung tumor development. This is the first report that absence of iNOS substantially decreases (>80%) chemically induced lung tumor development in mice. iNOS concentrations are high in lung tumors and in Clara cells but below detection limits of the immunoblotting technique in whole lung homogenates. The presence of iNOS in tumors and in a progenitor cell type (34) additionally implicates NO as a modulator of lung tumor development. The levels and cellular distribution of ecNOS and nNOS were unaffected by iNOS deficiency, making it unlikely that the NO produced by these enzymes was sufficient to compensate for the absence of iNOS. In terms of potential mechanisms by which iNOS modulates tumorigenesis, lung tumors isolated from iNOS-deficient mice contained 54% less VEGF than tumors derived from wild-type mice, implying that deficient neovascularization reduces lung tumor development in iNOS KO mice. NO regulation of lung tumor angiogenesis is additionally supported by localization of both iNOS and VEGF proteins in Clara cells, a lung tumor progenitor cell. iNOS is present in lung microadenomas as early as 3 weeks posturethane (data not shown), suggesting that iNOS is an early mediator of tumor development. Measurements of COX expression in tumors, lung PGE2 levels, and the lack of effect of iNOS deficiency on chronic inflammation revealed no interactions between NO and prostaglandin production and do not support NO involvement in BHT-induced chronic inflammation.

Similar to our observations with mouse lung tumors, human lung tumors express more NOS and VEGF than their surrounding tissue; elevated VEGF levels are associated with metastases and poor prognosis (12, 40, 41). Tumor hypoxia resulting from growth that overwhelms adequate oxygen sources is associated with neoplastic progression and stimulates angiogenesis (42). Hypoxia induces the hypoxia inducible factor-1α transcription factor, which in turn increases transcription of both iNOS and VEGF mRNA (42). iNOS-derived NO may mediate tumor growth by increasing tumor vascular permeability (43) as well as by enhancing VEGF expression to facilitate angiogenesis. In vitro studies using human lung cancer cell lines cocultured with endothelial cells show that decreasing NO reduces capillary formation (10). Lung colonization after tail vein injection of mouse melanoma cells is reduced, and tumors express less VEGF in mice lacking functional iNOS (44, 45). Tumor growth and VEGF mRNA expression are each reduced in s.c. B16-F1-derived tumors grown in iNOS (−/−) mice (19). Finally, s.c. tumors derived from injected colon AC cells that transgenically overexpress iNOS grow faster and are more vascularized than tumors derived from the parental cell line (46). Our experiments indicate that a deficiency in pulmonary NO reduces VEGF levels in mouse lung tumors. Others have found that VEGF induces NO release to promote capillary formation (47), and endothelial cell migration and growth (48). Thus, complicated feedback mechanisms affect inter-relationships between NO and VEGF to regulate tumor angiogenesis.

The epithelial cells at the periphery of lung tumors stained intensely...
Fig. 3. Immunohistochemistry of NOS enzymes in wild-type and iNOS (−/−) mice. Tumors from +/+ mice (A, C, and E) and iNOS (−/−) mice (B, D, and F). iNOS protein is strongly expressed (brown DAB staining) in tumors from a wild-type mouse (A), especially in cells at the tumor periphery (arrow). iNOS is not expressed in tumors from an iNOS KO mouse (B). iNOS deficiency does not affect expression of ecNOS, compared with tumors from wild-type mice (C and D). Staining of nNOS is similar in tumors from +/+ and iNOS KO mice (E and F). Five sections each from 3 wild-type and 3 iNOS KO mice were compared. Bar = 30 μm. Magnification = ×18.

Fig. 4. Immunohistochemistry of COX-1 and COX-2 in tumors from wild-type (A and C) and iNOS KO (B and D) mice. COX-1 (A and B) and COX-2 (C and D) protein are highly expressed in mouse lung tumors, and iNOS deficiency does not affect expression. Five sections each from 3 wild-type and 3 iNOS KO mice were compared. Bar = 30 μm. Magnification = ×18.
for iNOS, although iNOS was also detectable throughout the central portion of the tumor as well. This expression pattern in the tumor epithelial cells may indicate a role for NO in tumor cell proliferation as well as angiogenesis. Tumor epithelium and stroma express VEGF (40); tumor epithelial cell-derived NO may induce VEGF expression in both tumor and stroma. The decrease in tumor VEGF expression in iNOS (−/−) mice would be predicted to decrease lung tumor size. We did not see an effect on tumor size in these studies, possibly because of the early time point (16 weeks) at which the tumors were collected; the tumors are small (≤1 mm at the largest diameter) at this stage. Size differences between the tumors in wild-type and iNOS (−/−) mice might become significant if tumors were allowed to grow longer than 16 weeks, similar to findings of smaller s.c. B16-F1-derived tumors in iNOS (−/−) mice (19). The absence of iNOS did not affect the staining pattern, or intensity of ecNOS or nNOS in urethane-induced lung tumors. ecNOS has been implicated in VEGF-induced angiogenesis and vascular permeability in a cranial window model of neovascularization (49, 50), but these effects may be specific to the experimental system tested as compensatory changes in ecNOS caused by the absence of iNOS were not evident in our studies.

NO contributes to the development of tumors in other model systems including colon cancer, in which the absence of iNOS protein (5) or inhibition of iNOS activity (6, 7) reduces aberrant crypt foci formation and colon tumor formation. However, the role of NO in colon cancer may not be completely straightforward, as others have found that absence of iNOS promotes intestinal tumorigenesis (51). COX-2 is also a critical mediator in colon cancer (52), and an interaction between NO and COX-2 has been proposed for colon cancer as well as in various models of acute inflammation (6, 28, 30). iNOS and COX-2 immunostaining positively correlates with VEGF expression in human AC, suggesting that interactions between NO and prostaglandins stimulate angiogenesis (53). For this reason, we investigated NO-COX interactions in murine lung tumorigenesis. No difference in the cellular distribution or staining intensity of COX-1 or COX-2 in mouse lung tumors from wild-type and iNOS KO mice was detected. When this relationship was examined during BHT-induced pulmonary inflammation, a model that predisposes mice to lung tumorigenesis, the absence of iNOS did not reduce alveolar macrophage infiltration (Fig. 7) and did not change basal COX-1 or COX-2 expression or PGE2 production. This indicates that the loss of iNOS does not modulate COX activity during BHT-
induced pulmonary inflammation. The lack of iNOS-COX interaction was not because of compensatory NO production by ecNOS or nNOS as these protein levels were unaffected by iNOS deficiency.

iNOS (−/−) mice are resistant to carrageenan-induced pleurisy, which causes lung injury and inflammation (8). iNOS inhibition attenuates production of both NO and prostaglandins after administration of carrageenan into a s.c. air pouch in rats (30), supporting a role of NO in COX catalysis. Other studies have shown that prostaglandin production in lungs and peritoneal macrophages after LPS administration is blocked by inhibition of iNOS activity (28, 54). An important distinction is that we examined chronic inflammation, whereas the carrageenan and LPS models assessed acute inflammatory responses. Whereas chronic lung inflammation is a risk factor for murine lung tumorigenesis (21, 22, 25), our results suggest that elevations in alveolar macrophages are not suitable lung cancer biomarkers. Given the importance of iNOS in murine lung tumorigenesis, as evidenced by the inhibition of tumor formation in its absence, nitrosylated protein products from the macrophages may be appropriate for early lung cancer detection. Our data also implies that whereas iNOS is important for mouse lung tumor development, the mechanism for this involves pathways independent of those modulating BHT-induced chronic lung inflammation; this does not rule out a potential role of inflammation in iNOS-driven lung cancer.

The magnitude of the decrease in lung tumors that we observed (>80%) is rarely achieved with a single manipulation. The few other examples include sulindac sulfone (90% inhibition of lung tumorigenesis; Ref. 55), aerosolized budesonide (80–90% inhibition; Ref. 56), and prostacyclin elevation via PGIS overexpression (85–90% inhibition; Ref. 57). Sulindac sulfone regulates the cyclic GMP second messenger pathways used by NO (58, 59), and glucocorticoids can regulate iNOS expression, nitrotrosyamine formation, and exhaled NO in asthmatic patients (60). Thus, two of the most effective lung cancer chemopreventive agents described to date in this mouse model have in common the ability to regulate aspects of iNOS activity and/or expression. Collectively, these data suggest that iNOS is an important target for chemoprevention of lung AC. Selective iNOS inhibitors have been successfully used in the prevention of rodent colon cancer (6, 7), and our studies provide compelling rationale for evaluating iNOS inhibitors as chemopreventive agents in lung cancer.

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