Alveolar Hypoxia Promotes Murine Lung Tumor Growth through a VEGFR-2/EGFR-Dependent Mechanism

Vijaya Karoor1,3, Mysan Le4, Daniel Merrick2,3,4, Karen A. Fagan5, Edward C. Dempsey1,3,4, and York E. Miller1,3,4

Abstract

Patients with chronic obstructive pulmonary disease (COPD) are at an increased risk for the development of lung cancer, the mechanisms for which are incompletely understood. We hypothesized that the hypoxic pulmonary microenvironment present in COPD would augment lung carcinogenesis. Mice were subjected to chemical carcinogenesis protocols and placed in either hypoxia or normoxia. Mice exposed to chronic hypoxia developed tumors with increased volume compared with normoxic controls. Both lungs and tumors from hypoxic mice showed a preferential stabilization of HIF-2α and increased expression of VEGF-A, FGF2, and their receptors as well as other survival, proliferation, and angiogenic signaling pathways regulated by HIF-2α. We showed that tumors arising in hypoxic animals have increased sensitivity to VEGFR-2/EGFR inhibition, as chemoprevention with vandetanib showed markedly increased activity in hypoxic mice. These studies showed that lung tumors arising in a hypoxic microenvironment express increased growth, angiogenic, and survival signaling that could contribute to the increased lung cancer risk in COPD. Furthermore, the differential sensitivity of tumors arising in hypoxia to VEGFR-2/EGFR inhibition suggests that the altered signaling present in tumors arising in hypoxic lung might be therapeutically exploited in patients with underlying COPD. Cancer Prev Res; 1–11. ©2012 AACR.

Introduction

Lung cancer is the leading cause of cancer death in both men and women in the United States and the overall leading cause of cancer death in the world (1). The overall 5-year survival is approximately 15% and has only shown minimal improvement over the past 30 years (2). Tobacco smoke is the most important risk factor for lung cancer. Multiple epidemiologic studies have shown an increased risk for lung cancer in individuals with airflow obstruction or chronic bronchitis, even after correction for smoking intensity (3). Recent studies have shown an even stronger relationship between lung cancer and emphysema than airflow obstruction (4). The mechanisms by which chronic obstructive pulmonary disease (COPD) contributes to lung cancer risk are still unknown. In addition to an as yet poorly understood shared genetic susceptibility and smoking, other factors that may contribute are inflammation and oxidant damage (5). We hypothesized that alveolar hypoxia may also contribute to the increased risk for lung cancer in patients with COPD.

Patients with COPD often exhibit arterial hypoxemia, which reflects the admixture of blood perfusing well and poorly ventilated regions of the lung. The most common sites for lung cancer development are the upper lobes, which are also the most common sites for emphysematous change (6). Emphysematous blebs are poorly ventilated, but no data are available on the oxygen tension within these lesions. Tissue hypoxia within tumors has been shown to influence prognosis, by increasing angiogenesis and resistance to apoptosis, but we are not aware of studies of the effects of alveolar hypoxia on the development of lung cancer.

Members of the hypoxia-inducible factor (HIF) family of transcription factors regulate the cellular response to hypoxia and are likely to play a role in the increased cancer risk seen in COPD (7). It has been shown that the chronic inflammation characteristic of COPD can promote HIF stabilization by activation of NFκB (8). Mice that conditionally express both a nondegradable variant of HIF-2α and a mutant form of Kras (Kras<sup>G12D</sup>) in the lungs developed larger and more invasive tumors had an increased tumor burden and decreased survival compared with mice expressing only Kras<sup>G12D</sup> (9). Experiments with HIF-2α deletion unexpectedly showed an increase in tumor burden, associated with a decrease in the candidate tumor suppressor gene Sggb3a1, revealing the complexity of the relationship between HIF-2α expression and tumorigenesis, in
which either up- or downregulation from basal expression can have similar effects (10). We have evaluated the effects of hypobaric hypoxia on mouse lung carcinogenesis in response to 2 distinct chemical carcinogenesis models, both of which produce multiple primary lung tumors that do not commonly metastasize. Urethane is a complete carcinogen that acts by causing Kras mutations, whereas the 3-methylcholanthrene/butylated hydroxytoluene (MCA/BHT) initiation-promotion model also causes Kras mutations but is further dependent on alveolar inflammation caused by BHT (11, 12).

Materials and Methods

Mouse maintenance

Female FVB/N mice were purchased (Harlan) at ages 6 to 8 weeks and maintained in conventional caging in a controlled environment (12-hour light-dark cycle, chow and water ad libitum) in the Denver Veterans Affairs Medical Center Animal Care Facility. All animal procedures were approved by the Denver Veteran Affairs Medical Center Institutional Animal Care and Use Committee.

Carcinogenesis protocols

For urethane carcinogenesis, mice were injected intraperitoneally with 1 mg/gm urethane dissolved in saline. For MCA/BHT carcinogenesis, mice were injected intraperitoneally with 25 mg/gm MCA dissolved in corn oil, then injected weekly × 6 with BHT in corn oil (100 µg/gm on week one, then increased by 25 µg/gm weekly on weeks 2–6). One week after urethane or MCA injection, mice were placed into hypobaric hypoxia at a simulated altitude of 18,000 feet corresponding to an oxygen concentration of 11%. For experiments on vandetanib chemoprevention, mice were fed AIN-76A chow to which vandetanib was added to achieve a dose of 75 mg/kg (BW)/d, based on serial observation of chow consumption by the mice. This dose had previously been shown to be well tolerated and effective in reducing tumor numbers and volume, as well as VEGF signaling (13). Serial body weights were followed during the experiment. Mice were sacrificed by intraperitoneal injection of a mixture of pentobarbital (10 mg) and heparin (100 unit)/mouse in a 200 µL volume of saline. Blood was harvested by cardiac puncture and then lungs removed. Tumors were identified under a dissecting microscope, dissected free from the lung tissue, and measured using digital calipers. Tumors were either snap-frozen in liquid nitrogen or fixed in 10% buffered formalin and paraffin embedded. Microscopic analysis of selected samples was carried out to confirm tumor histology.

Chronic hypoxia

High-altitude chambers were used as a method to achieve chronic hypoxia: Animals were exposed to either normoxia (Denver altitude, 5,280 feet) or hypobaric hypoxia (simulated altitude, 18,000 feet, equivalent to FiO2 = 11%) for different time durations (14). Exposures were continuous with interruptions of less than 1 hour every 3 to 4 days for animal maintenance. Animals were weighed every week at this time. Food and water were provided ad libitum within the chamber.

Intermittent hypoxia

Intermittent hypoxia, to simulate sleep apnea, was achieved in an Oxycycler model A44 × 0 (Biospherix) chamber connected to a supply of O2 and N2 gases. Sensors measured O2 concentration, CO2 concentration (<0.01%), humidity (40%–50%), and temperature (22–24°C). Inflow of O2 and N2 into the chamber was controlled by a computer programmed to produce cycles of 2-minute room air–2-minute 10% O2 (corresponding to a PaO2 = 35 mm Hg). Mice were subjected to this schedule during the light period (07:00–19:00 for 20 weeks). Food and water were provided ad libitum within the chamber.

Western blotting

Lung homogenates were prepared in buffer containing 50 mmol/L Tris HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L EDTA, 2.5 mmol/L EGTA, 10% glycerol, 1% Nonidet P-40, 1 mmol/L dithiothreitol, 10 mmol/L β-glycerophosphate, 10 mmol/L NaF, 1 mmol/L sodium orthovanadate, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 10 µg/mL pepstatin A, and 1 mmol/L phenylmethylsulfonyl fluoride. The homogenates were centrifuged for 10 minutes at 10,000 rpm and the supernatant collected. Protein concentration was determined using the BCA protein assay. Proteins were separated on SDS-PAGE, transferred to nitrocellulose membranes (GE HealthCare). Membranes were blocked in PBS containing 0.1% Tween 20 and 1% bovine serum albumin for 1 hour. Membranes were incubated with primary antibodies overnight at 4°C and with secondary antibodies for 1 hour at room temperature. The sources of primary antibodies used are listed in Supplementary Materials.

Densitometry

A Bio-Rad gel scanner and densitometer (Gel DocXR with Quantity 1 program) was used to assess the intensity of the bands obtained by Western blots. Samples from normoxic and hypoxic mice were run on the same gel. The measured arbitrary density units were normalized to actin, and ratio of normoxia and hypoxia was calculated.

Cytokine antibody array

Lung lysates from urethane-treated mice exposed to normoxia or hypoxia for 2 weeks were incubated overnight at 4°C on a ChemiArray Mouse Antibody Array III Map (no. AA1003M; Chemicon International) containing antibodies against 62 different cytokines. Detection of protein signals was done according to the manufacturer's instructions. Membranes from normoxia and hypoxia were developed and exposed on the same film for comparison. Pooled samples from 3 mice were used for analysis.

Immunohistochemistry

Tumors and lungs were fixed in 10% buffered formalin overnight, embedded in paraffin, and cut in serial 4-µm
sections which were mounted on glass slides. All sections were pretreated with 10 mmol/L Tris EDTA, pH 9.0 for 20 minutes at 95°C. Sections were incubated with primary antibodies overnight at 4°C in a humidified chamber on a shaker. The sections were subsequently washed 3 times in TBS and incubated with secondary antibodies, either biotinylated anti-rat IgG or anti-rabbit IgG (both at 1:200 dilution; Vector Laboratories) for 1 hour. Tissues were then washed 3 times in TBS and incubated with horseradish peroxidase–conjugated Streptavidin (Vector Laboratories) for 30 minutes. Tissues were washed 3 times in TBS and developed in liquid DAB (Biogenex) for 5 to 10 minutes until a brown color appeared. Tissues were then washed 3 times in water, counterstained with modified Harris’ hematoxylin (Fisher Diagnostics) for 30 minutes, then washed 3 times in water, dipped in acid ammonia, washed 3 times in water, dehydrated in serial ethanol baths, cleared in Citri-Solv (Fisher Diagnostics), and mounted on slides. Primary antibodies included: 1:50 rat anti-mouse Ki-67 (Dako A/S); 1:100 rat anti-mouse CD34 (Santa Cruz Biotechnology). Controls included no primary antibody or same species IgG. Immunostaining was quantitated either by cell counting, for Ki-67 (cells with nuclear anti–Ki-67 immunostaining/400 cells), or the Chalkley grid method, for vessels (15). The Chalkley grid is mounted into a microscope eyepiece and displays points superimposed onto the image of a tissue section. Vessels are then quantitated by counting the numbers of points which are superimposed on CD34-positive structures with the appearance of capillaries and entering this data into an equation.

Statistical analysis
Data were analyzed using 2-sided, nonparametric t tests and Fischer exact test (one-sided) as appropriate. P values less than 0.05 were considered as significant.

Results

Hyoxia enhances tumor growth in urethane and MCA/BHT models of carcinogenesis
FVB/N mice (6–8 weeks old; 10–14 mice per group) were injected with urethane and allowed to recover for a week. Injected animals were maintained at normal ambient conditions or in hypobaric chambers at 18,000 feet (simulating 11% oxygen) for 11, 15, and 21 weeks. Tumors from hypoxic mice were larger than those from normoxic mice at 15 weeks (0.85 ± 0.01 vs. 2.5 ± 0.48 mm³, P < 0.001) and at 21 weeks (1.66 ± 0.21 vs. 4.94 ± 0.11 mm³, P < 0.001; Fig. 1A and B). Tumor volume increased proportionally with exposure time. Hyoxia did not have a significant effect on tumor multiplicity in the urethane model. Figure 1C shows tumor multiplicity and volume at 21 weeks from mice (10 mice per group) in normoxia or hypoxia in the MCA/BHT model. In this model, the tumor multiplicity was significantly lower in hypoxic mice (4.6 ± 0.87 vs. 2.69 ± 0.32 tumors/mouse, P < 0.05), whereas tumor volume was increased over that of normoxic mice (0.25 ± 0.06 vs. 1.33 ± 0.4 mm³, P < 0.005) at 21 weeks.

Microscopic analysis of tumors did not reveal alterations in tumor invasiveness, metastasis, or in histologic grade between groups in either model. In both models, mice in hypoxia initially lost weight and then gained weight at a similar rate to normoxic controls. Chronic hypoxia resulted in a modest increase in hematocrit in both models (Supplementary Fig. S1). As MCA/BHT is an initiation–promotion carcinogenesis model dependent on inflammation, we chose to further characterize the effects of hypoxia in the less complex urethane carcinogenesis model.

Intermittent hypoxia

As obstructive sleep apnea is a common condition that results in intermittent alveolar hypoxia, we tested the effects of chronic intermittent hypoxia on tumor growth in the urethane model. Mice (18 mice per group) were exposed to intermittent hypoxia for 21 weeks, lungs and tumors were dissected and tumor volume measured as described above. Chronic intermittent hypoxia did not affect tumor volume (0.88 ± 0.03 vs. 0.84 ± 0.26 mm³; P = ns) but reduced tumor numbers slightly (7.61 ± 0.44 vs. 6.11 ± 0.56 mm³; P ≤ 0.04; Fig. 1D).

Increased expression of HIF-2α and c-Myc in lungs and tumors from hypoxic mice

Hypoxia leads to stabilization of HIF transcription factors. We measured levels of HIF-1α and HIF-2α by immunoblotting of lysates from lungs and tumors obtained from urethane-treated mice in normoxia and hypoxia at 21 weeks. HIF-1α and HIF-2α were expressed in the lungs of urethane-treated mice in normoxia (Fig. 2A and C). In hypoxia, HIF-1α expression by densitometry was significantly (P < 0.005) reduced in hypoxic lungs and tumors. HIF-2α expression was 2-fold higher in hypoxic lungs and 2.7-fold higher in tumors from hypoxic mice compared with those in normoxia (P = 0.05; Fig. 2A and C). In comparison, samples from lungs and tumors of urethane-treated mice exposed to intermittent hypoxia did not show differences in levels of HIF-1α and HIF-2α expression (data not shown). c-Myc synergizes with HIF-2α in renal carcinoma and neuroblastoma to form more aggressive tumors (16). We examined expression of c-Myc and as shown in Fig. 2A, expression was higher in urethane-treated lungs (P < 0.005) and tumors under hypoxia (P < 0.05). Taken together, these data suggested that loss of HIF-1α expression coupled with an increase in HIF-2α and c-Myc may be important in hypoxia-induced tumor progression.

Increase in angiogenesis, proliferation, and expression of angiogenic factors in lungs and tumors from hypoxic mice

HIF-2α and c-Myc promote tumor growth by increasing proliferation and angiogenesis (17, 18). To determine whether increased angiogenesis occurred before measurable increases in tumor size, tumor sections from normoxic and hypoxic mice at 11, 15, and 21 weeks were stained with CD34 to identify vessels (Fig. 3A). The number of vessels was quantitated using the Chalkley grid method and average...
data from triplicate analyses are shown in Fig. 3B (15). Figure 3C depicts the correlation between tumor volume and microvascular density in normoxia and hypoxia. At 11 weeks, when there was no significant difference in tumor size between normoxic and hypoxic mice, there was significantly increased angiogenesis in hypoxia (Fig. 3B and C). This was accompanied by an increase in Ki67 stain (Supplementary Fig. S2).

Angiogenesis is regulated by various growth factors that are released from an extracellular matrix bound form by matrix metalloprotease (MMP; refs. 19–21). We analyzed lungs and tumors for the expression of angiogenic growth factors and levels of MMPs. Hypoxia increased the expression of the angiogenic growth factors VEGF-A and C as well as FGF-2 in both lungs and tumors (Fig. 3D and E). MMP-2 expression was significantly upregulated in both urethane-treated lungs and tumors under hypoxia, whereas MMP-9 expression was significantly increased in tumors only.

**Hypoxia enhances the expression of inflammatory cytokines in lung**

Cytokines play an important role in inflammatory airway diseases and have been shown to be upregulated in COPD lungs and cancer (22–24). Inflammation contributes to cancer progression by modulating proliferation and survival of malignant cells, promoting angiogenesis and metastasis, and decreasing adaptive immunity (25–29). Recent
studies have shown that hypoxia increases the pulmonary expression of chemokines and cytokines important for the infiltration of inflammatory cells and endothelial progenitor cell recruitment (30). We analyzed lung lysates for the expression of inflammatory cytokines using an antibody array. Figure 3E shows the levels of select cytokines after 2 weeks of hypoxia or normoxia. Hypoxia increased the expression of multiple proinflammatory cytokines in lungs from urethane-treated mice. These results suggested that hypoxia promotes a proinflammatory milieu in the lung, which likely contributes to tumor progression.

Hypoxia increases the levels of growth factor receptors in lungs and tumors

To further analyze the mechanism of enhanced tumor growth in hypoxia, we measured levels of various growth factor receptors known to promote tumor growth and angiogenesis. As shown in Fig. 4A and B, lungs and tumors from hypoxic mice showed an increase in levels of EGFR, FGFR2, and PDGFR. Levels of additional receptors that regulate growth and maintenance of vasculature were analyzed in lungs and tumors (Fig. 4C and D). Under hypoxic conditions, lungs from urethane-treated mice had higher levels of Tie1, Tie2, VEGFR1, and VEGFR2. In tumors, hypoxia increased levels of VEGFR2. Levels of Tie1, Tie2, and VEGFR1 were lower in hypoxic tumors. As VEGFR1 acts in some situations as a decoy receptor decreasing signaling through VEGFR2, this decrease may augment VEGFR2 activity in tumors from hypoxic animals. Our results suggested that VEGFR2 might play a critical role in the increased tumor growth observed in hypoxic mice.

Effects of hypoxia on epithelial-to-mesenchymal transition markers

To determine whether hypoxia promoted the expression of genes important in epithelial-to-mesenchymal transition (EMT), we measured expression levels of E-cadherin, N-cadherin, Snail, β-catenin, p120 catenin, α-smooth muscle actin, vimentin, and fibronectin in lungs and tumors from normoxic and hypoxic mice. In lungs treated with urethane, hypoxia caused a statistically significant decrease in p120 catenin. Levels of Snail, vimentin, and fibronectin were significantly higher in hypoxic lungs (Fig. 5A and B). Tumors from hypoxic mice showed loss of E-cadherin, β-catenin, p120 catenin and an increase in the expression of vimentin and Snail (Fig. 5A and B). These results showed that hypoxia regulates the expression of proteins necessary for EMT both in lungs and, more prominently, in tumors.

Vandetanib chemoprevention under normoxic and hypoxic conditions

As hypoxic tumors showed a striking upregulation of both VEGF-A and VEGFR-2 (as well as other signaling pathways), we hypothesized that chemoprevention with vandetanib, a combined VEGFR/EGFR tyrosine kinase inhibitor, might be differentially effective in chemoprevention under hypoxic conditions. One week after urethane
Figure 3. Increased expression of angiogenic factors and inflammatory cytokines in mice exposed to hypoxia. Tumor sections obtained from 11-, 15-, and 21-week urethane-treated mice exposed to normoxia or hypoxia were stained with anti-CD34 to identify vessels. A, CD34 stain and a quantitative representation of vessels is shown in B. C, the relationship between tumor volume and mean vessel counts is expressed graphically, showing that at a time point (11 weeks) where tumor volume is not increased, mean vessel count is nearly doubled in hypoxia. Lung and tumor lysates obtained from mice exposed to normoxia or hypoxia after urethane were analyzed for the expression of proangiogenic factors by Western blotting and are shown in D. β-Actin was used as a loading control. E, a graphical representation of expression levels in lungs and tumors (n = 3 for each treatment). Lysates of lungs of urethane-treated mice exposed to normoxia or hypoxia for 2 weeks were analyzed for expression of inflammatory cytokines using an antibody array from Millipore and select cytokines from pooled samples (n = 3) for each treatment are shown in F. Data shown is mean ± SEM and * indicates P < 0.05 for comparisons between normoxia and hypoxia.
injection, mice (6–9 mice per group) were given AIN-76A chow containing vandetanib to achieve a dose of 75 mg/kg (BW)/d or AIN-76A without vandetanib, previously shown to be effective in inhibition of VEGFR-2 and EGFR signaling, as well as chemopreventive, then maintained in either normoxia or hypoxia (13). Vandetanib treatment resulted in a 33% reduction in tumor incidence in normoxia and completely prevented tumors in hypoxia (P ≤ 0.01, Fischer exact test, one sided; Fig. 6A). As tumor incidence was 0% in hypoxic conditions, the effect on tumor multiplicity and volume, as reported in our earlier article, could not be calculated. Thus, vandetanib is more highly chemopreventive under hypoxic conditions.

Vandetanib decreases accumulation of HIF-2α and expression of c-Myc and growth factor receptors in hypoxic mice

To identify targets of vandetanib, we probed lung lysates from treated and untreated normoxic and hypoxic mice for expression of select proteins that were significantly altered in hypoxic conditions. As seen in Fig. 6B–G, vandetanib significantly inhibited expression of HIF-2α, c-Myc, VEGF, SDF-1, and EGFR, PDGFR, VEGFR2, FGFR2 growth factor receptors in hypoxic mice.

Discussion

Only a subset of tobacco smokers develops COPD. COPD has been repeatedly shown to be associated with an increased risk of lung cancer, even after adjustment for smoking (31). More recently, the presence of emphysema on CT scan has been more strongly associated with lung cancer risk than is airflow obstruction on spirometry (4, 32). The mechanisms by which COPD confers an increased lung cancer risk are not clear, although a common genetic susceptibility, increased inflammation, and oxidant radical exposure all may play a role. We hypothesized that alveolar hypoxia might be critical in the increased risk of lung cancer seen in COPD. We believe that this has not been previously investigated.

Using 2 chemical carcinogenesis models and exposure of mice to hypobaric hypoxia, we showed that alveolar hypoxia consistently promotes lung tumor growth, commonly considered a marker of tumor progression. In both models, mice were allowed to recover for a week after the completion of carcinogenesis protocol before being placed in hypoxia. This was planned so as to minimize any potential effect of the hypoxic environment on metabolism of the carcinogen. Mice kept under hypoxic conditions initially lost weight, but after a week gained weight at the same rate as normoxic mice. Experimental weight loss typically has a suppressive effect on carcinogenesis and tumor progression, but we cannot be sure that hypoxia-induced weight loss might not have different effects, although this seems unlikely (33).

Alveolar hypoxia did not increase tumor multiplicity in these models and indeed resulted in a statistically significant decrease in tumor multiplicity in the MCA/BHT model, as well as in intermittent hypoxia. We do not understand the mechanisms of suppression of tumor multiplicity in these models and indeed resulted in a statistically significant decrease in tumor multiplicity in the MCA/BHT model, as well as in intermittent hypoxia. We do not understand the mechanisms of suppression of tumor multiplicity in the MCA/BHT and intermittent hypoxia models, but doubt that an effect on metabolism of the carcinogens urethane or MCA would have been operant, as mice were not placed
in hypoxia until a week after administration of either carcinogen.

The hypobaric hypoxia exposure we used resulted in a decrease in PaO2 (arterial PO2) from 66 to 40 mm Hg (corresponding to a change in O2 saturation from 92% to 71%) in experiments conducted in Denver, CO (elevation 5,280 feet), similar to PaO2 values sometimes seen in COPD patients without O2 supplementation (14). PaO2 is a reflection of the mixture of pulmonary venous blood that has perfused either well- or poorly ventilated regions of the lung. Thus, the presence of even a modestly reduced PaO2 of 50 (commonly seen in COPD patients in Denver) would imply that the less well-ventilated alveoli of that patient’s lung are exposed to alveolar oxygen tensions well below 50.

Therefore, we believe that the experimental conditions of this model do simulate alveolar hypoxia that is present in the lungs of patients with COPD. Both hypobaric and normobaric hypoxia models are used to assess the cardio-pulmonary effects of hypoxia. No major differences in the physiologic response to chronic hypoxia have been shown between the 2 models (34).

Sleep apnea is another common cause of hypoxia, in which hypoxia is intermittent, rather than continuous. There is no known association between sleep apnea and lung cancer. We found that an intermittent model of hypoxia did not reproduce the stimulatory effect of continuous hypoxia on lung tumor growth. In addition, intermittent hypoxia did not result in increased lung and tumor levels of HIF-2α, as seen in continuous hypoxia. We speculate that upregulation of HIF-2α may be necessary for the tumor growth stimulatory effect of chronic hypoxia.

Exposure to hypoxia significantly increased tumor volume, proliferation, and angiogenesis. The HIF transcription factors are upregulated by hypoxia and increase the expression of multiple growth and angiogenic factors. We found a preferential stabilization of HIF-2α accompanied by a decrease in HIF-1α expression both in lungs and tumors from hypoxic mice. HIF-2α overexpression in a conditionally expressed mutant KrasG12D model of lung carcinogenesis resulted in larger tumors, similar to our findings with alveolar hypoxia (9).

In patient non–small cell lung cancer samples, HIF-1α and HIF-2α overexpression is frequently observed and correlates with the expression of angiogenic factors and poor outcome (35).

To define mechanisms by which hypoxia promotes tumor growth, we analyzed pathways important in proliferation, angiogenesis, and survival, major hallmarks of cancer (36). Angiogenesis is essential for tumor growth and progression (37). The balance of proangiogenic and antiangiogenic factors and degradation of extracellular matrix in the tumor microenvironment are important steps in angiogenesis (38). Lungs and tumors from hypoxic mice showed higher levels of proangiogenic factors FGF2, VEGF-A and increases in MMP-2 and MMP-9. VEGFA and MMP-9 were selectively upregulated in hypoxic tumors, suggesting an important role.

Angiogenesis is regulated by vascular growth factor receptors that include Tie receptors, predominantly involved in vessel homeostasis, and VEGF receptors, which are implicated predominantly in angiogenesis. Mice with homozygous deficiency in any of these receptors show defects in vasculogenesis and are nonviable during embryogenesis. Lungs of hypoxic mice treated with urethane showed higher levels of Tie1 and 2 and VEGFR1 and 2. Tumors from hypoxic mice had decreased VEGFR1, Tie1 and 2 and increased VEGFR2 levels compared with those from normoxic mice. In tumors from hypoxic mice, downregulation of VEGFR1 and Tie2 receptors, both important for vessel maturation, might lead to abnormal leaky vessels as found in aggressive tumors.

Analysis of additional growth factor receptor levels in lungs and tumors revealed that hypoxic mice significantly overexpressed EGFR, FGFR2, and PDGFR. To further characterize the effect of hypoxia on pathways important in cell proliferation, survival, apoptosis, and migration, we examined downstream signaling from these receptors. Mitogen-activated protein kinases, including Erk, p38 kinase, and JNK, are known to play important roles in cell proliferation,
migration, and survival and are deregulated in many cancers (39, 40). Hypoxia caused a significant increase in active Erk and a decrease in active JNK in lung (Supplementary Fig. S3A and B). Tumors from hypoxic mice showed a selective activation of JNK. The JNK/c-Jun/AP-1 pathway is important in mediating oncogenic Ras function in lung carcinoma cells (41).

Hypoxia increases cell survival by upregulation of anti-apoptotic factors, thereby making tumors resistant to therapy (42). In hypoxic lung and tumors, we found higher levels of p-Akt, c-IAP-1, and Bcl2 (Supplementary Fig. S3C and D).

We also investigated effects of hypoxia on pathways important in EMT (43). E-cadherin expression was decreased in tumors of hypoxic mice. Hypoxia decreased levels of β-catenin and p120 catenin in urethane-treated lungs. Tumors from hypoxic mice had lower levels of E-cadherin and both β-catenin and p120 catenin.

Figure 6. Vandetanib decreases tumor incidence and accumulation of HIF-2α, expression of c-Myc and growth factor receptors in the lung of hypoxic mice. A, tumor incidence in mice (6–9 mice per group) treated with or without vandetanib 75 mg/kg for 15 weeks in normoxia or hypoxia. Vandetanib completely suppresses tumor development in hypoxia but not in normoxia (P = 0.01, Fischer exact test, one-sided). The effect of vandetanib on abundance of HIF1α, HIF2α, c-Myc, angiogenesis factors, and their receptors in normoxia and hypoxia is shown in B–G. Data shown are mean ± SEM and * indicates P ≤ 0.05 for comparisons between untreated normoxia and vandetanib treated in each group.
studies have shown a role for Snail not only in EMT but also in promoting tumor growth by increasing cytokine-mediated angiogenesis. Snail has been shown to be associated with poor prognosis (44). In urethane-treated lungs and tumors, hypoxia significantly increased Snail expression with a decrease in E-cadherin expression in tumors only.

Chemokines and cytokines have been implicated in lung carcinogenesis by recruiting inflammatory and endothelial progenitor cells to the lungs (45). In urethane-treated mice exposed to hypoxia, there were increased levels of chemokines, including G-CSF and SDF-1, which are important in progenitor cell recruitment (46). Interestingly, a predominance of factors important in T-cell function and macrophage recruitment was observed (47). These observations suggested that hypoxia promotes a chemokine mediated proinflammatory milieu in the lungs, which likely contributes to carcinogenesis.

The quantitative changes induced by hypoxia in expression of multiple proteins described above are summarized in Supplementary Table S1. In addition to the expression changes in urethane-treated lung and urethane-induced tumors, expression changes induced by hypoxia alone are summarized.

Due to the increase in VEGFA, VEGFR-2, and EGFR seen in tumors from hypoxic mice, we hypothesized that inhibition of these pathways might be particularly effective in chemoprevention under hypoxic conditions. Vandetanib is a receptor tyrosine kinase inhibitor primarily targeting VEGFR-2 and EGFR that we have previously shown to be a potent chemopreventive agent in normoxia, likely due to its VEGFR-2 targeted activity, as the EGFR inhibitor gefitinib is inactive in these models (48). Vandetanib significantly decreased tumor development in normoxia and completely prevented it in hypoxia. Simple clinical features, such as smoking history, gender, and ethnicity have proved to be useful predictors of response to EGFR tyrosine kinase inhibitors, and we speculate that clinical features associated with alveolar hypoxia, such as emphysema on CT, may also have predictive value, particularly for agents that interfere with signaling pathways upregulated by hypoxia. The differential sensitivity to vandetanib suggests that the presence of emphysema or airflow obstruction might be a useful predictive marker of sensitivity to selected targeted agents.

These results show that alveolar hypoxia increases tumor growth, associated with increased tumor proliferation and angiogenesis. Although tumor multiplicity is not increased in alveolar hypoxia and is suppressed in the MCA/BHT model, it is biologically plausible that hypoxia-augmented progression of premalignant or clinically unapparent malignant lesions to apparent lung cancer may contribute to the increased lung cancer risk seen in COPD. Alveolar hypoxia is associated with increased HIF-2α protein, as well as increases in growth factors, growth factor receptors, survival factors, and signaling cascades. Administration of the dual VEGFR2/EGFR tyrosine kinase inhibitor, vandetanib, has markedly increased efficacy in the chemoprevention of tumors arising under hypoxic conditions. Lung cancer arising in patients with COPD may have increased response to agents targeting pathways upregulated by hypoxia, such as VEGFA/VEGFR-2.

Disclosure of Potential Conflicts of Interest

Y.E. Miller is a co-inventor of a patent for the use of prostacyclin analogs for the prevention of cancer. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: V. Karoor, M. Le, E.C. Dempsey, Y.E. Miller Development of methodology: V. Karoor, M. Le, E.C. Dempsey, Y.E. Miller Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): V. Karoor, M. Le, D. Merrick, K.A. Fagan, Y.E. Miller Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V. Karoor, M. Le, D. Merrick, E.Y. Miller Writing, review, and/or revision of the manuscript: V. Karoor, M. Le, D. Merrick, K.A. Fagan, E.C. Dempsey, Y.E. Miller Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): M. Le

Study supervision: M. Le, Y.E. Miller

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