Alveolar Hypoxia Promotes Murine Lung Tumor Growth Through A VEGFR-2/EGFR Dependent Mechanism.

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Abstract

Patients with chronic obstructive pulmonary disease (COPD) are at 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 to 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 demonstrated 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 demonstrate that lung tumors arising in an 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.
Introduction

Lung cancer is the leading cause of cancer death in both men and women in the US 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 thirty years (2). Tobacco smoke is the most important risk factor for lung cancer. Multiple epidemiological 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 demonstrated 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 demonstrated 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 non-degradable variant of HIF-2α and a mutant form of Kras (Kras$^{G12D}$) in the lungs developed larger
and more invasive tumors, had an increased tumor burden and decreased survival compared with mice expressing only $Kras^{G12D}$ (9). Experiments with HIF-2α deletion unexpectedly demonstrated an increase in tumor burden, associated with a decrease in the candidate tumor suppressor gene Scgb3a1, revealing the complexity of the relationship between HIF-2α expression and tumorigenesis, in which either up or down regulation from basal expression can have similar effects (10). We have evaluated the effects of hypobaric hypoxia on mouse lung carcinogenesis in response to two 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, Indianapolis) at ages 6-8 weeks and maintained in conventional caging in a controlled environment (12 h 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 IACUC.

**Carcinogenesis Protocols** For urethane carcinogenesis, mice were injected i.p. with 1 mg/gm urethane dissolved in saline. For MCA/BHT carcinogenesis, mice were injected i.p. with 25 μg/gm MCA dissolved in corn oil, then injected weekly x 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 demonstrated 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 i.p. 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 performed 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, 5280 feet) or hypobaric hypoxia
(simulated altitude, 18,000 feet, equivalent to FiO2 = 11%) for different time durations(14).
Exposures were continuous with interruptions of <1 h every 3–4 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 A44X0 (Biospherix, Redfield, NY) 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 min room air–2 min 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 wks).
Food and water were provided ad libitum within the chamber.
**Western Blotting** Lung homogenates were prepared in buffer containing 50 mM Tris HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, 1% Nonidet P-40, 1 mM dithiothreitol, 10 mM β-glycerophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged for 10 min 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, San Diego, CA). Membranes were blocked in PBS containing 0.1% Tween 20 and 1% BSA for 1 h. Membranes were incubated with primary antibodies overnight at 4 °C, and with secondary antibodies for 1 h at room temperature. The sources of primary antibodies used are listed in Supplemental 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 two 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 three 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 mM Tris EDTA, pH 9.0 for 20’ 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 3x in TBS and incubated with secondary antibodies, either biotinylated anti rat IgG or anti rabbit IgG (both at 1:200 dilution, Vector Laboratories, Burlingame, CA) for one hour. Tissues were then washed 3x in TBS and incubated with HRP conjugated Streptavidin (Vector Laboratories) for 30’. Tissues were washed 3x in TBS and developed in liquid DAB (Biogenix, San Ramon, CA) for 5-10’ until a brown color appeared. Tissues were then washed 3x in water, counterstained with modified Harris’ hematoxylin (Fisher Diagnostics, Middletown, VA) for 30’, then washed 3x in water, dipped in acid ammonia, washed 3x in water, dehydrated in serial ethanol baths, cleared in CitriSolv (Fisher Diagnostics) and mounted on slides. Primary antibodies included: 1:50 rat anti mouse Ki-67 (Dako A/S, Glostrup, Denmark); 1:100 rat anti mouse CD34 (Santa Cruz Biotechnology, Santa Cruz, CA. 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 two-sided, non-parametric t-tests and Fischer’s exact Test (one-sided) as appropriate. p values <0.05 were considered as significant.

Results

Hypoxia enhances tumor growth in urethane and MCA/BHT models of carcinogenesis. FVB/N mice (6-8 wks old; 10-14 mice/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≤
Tumor volume increased proportionally with exposure time. Hypoxia 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/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) while tumor volume was increased over that of normoxic mice (0.25 ± 0.06 vs 1.33 ± 0.4 mm³, p ≤ 0.005) at 21 wks. Microscopic analysis of tumors did not reveal alterations in tumor invasiveness, metastasis or in histological 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. (Figure 1, Supplement) 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/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) (Figure 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 (Figure 2A and 2C). In hypoxia, HIF-1α expression by
densitometry was significantly (p<0.005) reduced in hypoxic lungs and tumors. HIF-2α expression was two-fold higher in hypoxic lungs and 2.7 fold higher in tumors from hypoxic mice compared to those in normoxia (p = 0.05) (Figure 2A and 2C). 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 Figure 2A, expression was higher in urethane treated lungs (p <0.005) and tumors under hypoxia (p<0.05). Taken together, these data suggest 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 prior to 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 (Figure 3A). The number of vessels was quantitated using the Chalkey grid method and average data from triplicate analyses is shown in Figure 3B (15). Figure 3C depicts the correlation between tumor volume and microvascular density in normoxia and hypoxia. At 11 weeks, when there is no significant difference in tumor size between normoxic and hypoxic mice, there was significantly increased angiogenesis in hypoxia (Figure 3B and C). This was accompanied by an increase in Ki67 stain (supplement Figure 2).

Angiogenesis is regulated by various growth factors which are released from an extracellular matrix bound form by matrix metalloprotease (MMPs) (19-21). We analysed 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 (Figure 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 analysed 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 pro-inflammatory cytokines in lungs from urethane treated mice. These results suggest that hypoxia promotes a pro-inflammatory 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 Figure 4A and B, lungs and tumors from hypoxic mice show an increase in levels of EGFR, FGFR2 and PDGFR. Levels of additional receptors that regulate growth and maintenance of vasculature were analysed in lungs and tumors (Figure 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 suggest that VEGFR2 might play a critical role in the increased tumor growth observed in hypoxic mice.

**Effects of hypoxia on epithelial to mesenchymal transition (EMT) markers** To determine whether hypoxia promoted the expression of genes important in 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 (Figure 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 (Figure 5A and B). These results demonstrate 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 injection, mice (6-9 mice/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’s exact test, one-sided) (Figure 6A). As tumor incidence was 0% in hypoxic conditions, the effect on tumor multiplicity and volume, as reported in our earlier
manuscript, 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 hypoxia. As seen in Figure 6 B-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 demonstrated 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 two chemical carcinogenesis models and exposure of mice to hypobaric hypoxia, we demonstrated 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 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 utilized resulted in a decrease in PaO\(_2\) (arterial PO\(_2\)) from 66 to 40 mm Hg (corresponding to a change in O\(_2\) saturation from 92% to 71%) in experiments conducted in Denver, CO (elevation 5,280 feet), similar to PaO\(_2\) values sometimes seen in COPD patients without O\(_2\) supplementation (14). PaO\(_2\) is a reflection of the mixture of pulmonary venous blood which has perfused either well or poorly ventilated regions of the lung. Thus, the presence of even a modestly reduced PaO\(_2\) 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 cardiopulmonary effects of hypoxia. No major differences in the physiologic response to chronic hypoxia have been demonstrated between the two 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-2\(\alpha\), 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 \( \text{Kras}^{G12D} \) 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 analysed pathways important in proliferation, angiogenesis and survival, major hallmarks of cancer (36). Angiogenesis is essential for tumor growth and progression (37). The balance of pro-angiogenic and anti-angiogenic 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 pro-angiogenic factors FGF2, VEGF-A and increases in MMP-2 and MMP-9. VEGF-A and MMP-9 were selectively upregulated in hypoxic tumors suggesting an important role.

Angiogenesis is regulated by vascular growth factor receptors which 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 to those from normoxic mice. In tumors from hypoxic mice, down regulation 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. MAPKs, 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 (Figure 3A and B, Supplement). 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 (Supplement, Figure 3C 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- and p120 catenins. Recent 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 suggest that hypoxia promotes a chemokine mediated pro-inflammatory 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 Table 1, supplement. 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 VEGR-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 demonstrate that alveolar hypoxia increases tumor growth, associated with increased tumor proliferation and angiogenesis. While 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.

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Figure legends:

Figure 1. Hypoxia increases tumor volume in urethane and MCA/BHT models of lung tumorigenesis. Mice were injected with urethane (10-14 mice/group) or MCA/BHT (10 mice/group) and exposed to hypobaric hypoxia (18,000 ft) or Denver altitude for 11, 15 and 21 weeks (urethane exposed mice) or 21 weeks (MCA/BHT exposed mice). A representative picture of lungs with tumors from normoxic and hypoxic mice at 15 weeks is shown in Panel A. Panel B shows tumor multiplicity and volume from urethane treated mice at 11, 15 and 21 weeks of hypoxic exposure. Panel C shows data obtained from mice treated with MCA/BHT at 21 weeks. Panel D shows effect of intermittent hypoxia on tumor number and volume in the urethane model of carcinogenesis. Data shown is mean ± SEM and * indicates p ≤ 0.05 for comparisons between normoxia and hypoxia.

Figure 2. HIF-2α and c-Myc are upregulated in lungs and tumors of mice exposed to hypoxia. Lysates of lungs and tumors obtained from urethane treated mice exposed to normoxia or hypoxia were analysed for the expression of HIF-1α, HIF-2α and c-myc transcription factors by western blotting. β-actin was used as a loading control. Panel A shows expression levels in chronic hypoxia and Panel B shows HIF-2α stain in normoxic and hypoxic lung. Panel C shows the fold change compared to normoxia of HIF-1α, HIF-2α and c-Myc in hypoxia for different conditions. Data shown is mean ± SEM and * indicates p ≤ 0.05 for comparisons between normoxia and hypoxia.

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. Panel A shows CD34 stain and a quantitative representation of vessels is shown in Panel B. In Panel C, the relationship between tumor volume and mean vessel counts is expressed graphically,
demonstrating 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 analysed for the expression of pro-angiogenic factors by Western blotting and are shown in Panel D. β-actin was used as a loading control. Panel E shows 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 two weeks were analysed 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 Panel F. Data shown is mean ± SEM and * indicates p ≤ 0.05 for comparisons between normoxia and hypoxia.

Figure 4. Increased growth factor receptor expression in lungs and tumors of hypoxic mice. Lysates from lungs obtained from urethane treated mice exposed to normoxia or hypoxia and tumors from normoxic and hypoxic mice were analysed for growth factor receptor expression by Western blotting. β-actin was used as a loading control. Panel A shows levels of EGFR, PDGFR and FGFR2 and Panel B shows a graphical representation of levels for various treatments. Panel C shows VEGFR1, VEGFR2, Tie1 and Tie2 and Panel D shows the graphical representation of the levels for various treatments. Data shown is mean ± SEM and * indicates p ≤ 0.05 for comparisons between normoxia and hypoxia (n=3 for each treatment).

Figure 5. Increased expression of mesenchymal markers in lungs and tumors of hypoxic mice. Lungs and tumors from urethane treated normoxic and hypoxic mice were analysed for expression of mesenchymal markers. Panel A shows levels of E-cadherin, N-cadherin, Snail, β-catenin, p120 catenin, α-Smooth muscle actin, vimentin and fibronectin. Panel B shows a graphical representation of levels for various treatments. Data shown is mean ± SEM and * indicates p ≤ 0.05 for comparisons between normoxia and hypoxia.
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. Panel A shows tumor incidence in mice (6-9 mice/group) treated with or without vandetanib 75 mg/Kg for 15 wks in normoxia or hypoxia. Vandetanib completely suppresses tumor development in hypoxia, but not in normoxia (p = 0.01, Fischer's 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 panels B-G. Data shown is mean ± SEM and * indicates p ≤ 0.05 for comparisons between untreated normoxia and vandetanib treated in each group.
FIGURE 1

A

B

C

D

FIGURE 1

A

B

C

D
FIGURE 4

A

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<td>EGFR</td>
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<td>FGFR</td>
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<td>PDGFR</td>
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<td>β-actin</td>
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</table>

B

Fold Change in Hypoxia

- Lung
- Tumor

EGFR | FGFR2 | PDGFR

C

<table>
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<tr>
<th>Lung</th>
<th>Tumor</th>
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<tbody>
<tr>
<td>Normoxia</td>
<td>Hypoxia</td>
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<tr>
<td>VEGFR1</td>
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<tr>
<td>VEGFR2</td>
<td></td>
</tr>
<tr>
<td>Tie 1</td>
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<tr>
<td>Tie 2</td>
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<td>β-actin</td>
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</table>

D

Fold Change in Hypoxia

- VEGFR1
- VEGFR2
- Tie 1
- Tie 2
Alveolar Hypoxia Promotes Murine Lung Tumor Growth Through a VEGFR-2/EGFR Dependent Mechanism

Vijaya Karoor, Mysan Le, Daniel Merrick, et al.


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