Transgenic mice overexpressing hepatocyte growth factor in the airways show increased susceptibility to lung cancer

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Several studies have suggested a possible role of the hepatocyte growth factor (HGF)/c-Met system in lung tumor development and progression. Extent of expression of both HGF and c-Met have been shown to be negative prognostic indicators of survival and recurrence in non-small-cell lung cancer, especially adenocarcinoma. To further define a role for HGF in lung cancer development and growth, we have generated transgenic mice that overexpress HGF in the airway epithelium. HGF transgenic and wild-type mice were exposed to the tobacco carcinogen, nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), or saline control and killed 10–38 weeks after exposure. Lungs were formalin inflated, paraffin embedded and sectioned. It was verified that the HGF transgene was expressed only in the lungs of transgenic mice. The transgenic mouse lung histology exhibited congestion in the alveolar spaces, excess production of blood vessels and a convoluted pattern of airways with wide bifurcations. The number of lung tumors from NNK-treated transgenic animals versus the number of lung tumors from NNK-treated wild-type animals was significantly higher (P = 0.0001, Poisson regression). The percentage of animals with tumors was 75% in the transgenic group compared with 48.8% in the wild-type group. The main effect was an increase in tumor multiplicity; average size of tumors was not different between the groups. Additionally, the tumors that arose in the transgenic mice contained increased HGF protein compared with tumors from the wild-type mice. These results indicate that lung carcinogenesis induced by a tobacco carcinogen is enhanced by expression of the HGF transgene. This model recapitulates the phenotype of aggressive lung adenocarcinoma that overexpresses HGF and will be useful in evaluating antitumor agents that target either the HGF/c-Met pathway or downstream effects such as angiogenesis or invasion.

Introduction

Lung cancer is currently the leading cause of cancer mortality in both men and women in the US. The 5 year survival rate for all stages of lung cancer combined is only 15% and for late-stage disseminated disease is <5% (1). Lung cancer patients currently have few therapeutic options and new targeted approaches are needed to improve long-term survival. Animal models remain pivotal to cancer drug discovery and development, particularly in such a complex and inaccessible organ as the lung. Novel preclinical models that mimic aggressive lung cancer are necessary to test potential therapeutic targets for lung cancer treatment.

Several studies have suggested a role of hepatocyte growth factor (HGF), also known as scatter factor, and its receptor, c-Met, in the development and progression of many human malignancies, including lung cancer (2–4). HGF is primarily a paracrine factor produced by mesenchymal cells (5), particularly fibroblasts, although cancerous epithelial cells may secrete HGF under some circumstances (6–9). Thus, HGF can signal through both paracrine and autocrine mechanisms. The c-Met receptor is expressed universally by both epithelial and endothelial cells (5). The well-characterized activities of the HGF/c-Met signaling pathway, including proliferation, invasion, angiogenesis and anti-apoptotic effects, provide examples of the different mechanisms by which this pathway is involved in tumor development and progression.

We have demonstrated previously that the HGF/c-Met pathway is important for in vivo human lung tumor growth. In this regard, we have reported a 3-fold stimulation of intraepithelial tumor volume when recombinant HGF (rHGF) was injected into human airway segments that contained lung adenocarcinoma cells implanted in scid mice (10). Additionally, the number of tumors growing in the sub-mucosal spaces of the airway xenografts increased from 11 to 67% when treated with rHGF (10). In a separate study, lung adenocarcinoma HGF protein levels were examined as a prognostic indicator in 56 patients (11). This analysis revealed that elevated HGF content of tumors defined a subset of Stage I patients who recurred and had shortened survival times. Furthermore, patients whose T stage was >1 and had elevated HGF rapidly recurred and died from their disease (11). Thus, in non-small-cell lung cancer patients, HGF could be a useful prognostic indicator for both early and advanced stage patients. Higher HGF levels within the tumor at the time of resection may be an indication that more malignant cells have migrated to other sites, increasing the probability of recurrence. Additionally, the HGF/c-Met pathway has proven to be an effective therapeutic target for lung cancer treatment both in vitro and in vivo (12,13).

To recapitulate the phenotype of aggressive lung cancer containing elevated HGF, we created a transgenic mouse strain that overexpresses human HGF in the airway epithelium under control of the rat Clara cell secretory protein (CCSP) promoter. Bell et al. (14) have demonstrated that this human HGF cDNA
sequence under control of the albumin promoter can promote liver tumors in mice. CCSP is an abundant 10 kDa secreted protein expressed in the non-ciliated airway epithelial (Clara) cell of the conducting small airways (15,16). Clara cells are believed to be the cells of origin for lung adenocarcinomas (17). Clara cells also function as the progenitor cells of epithelial differentiation in the developing lung. The CCSP promoter has been used previously to direct expression of the human growth hormone (hGH) gene to the airways (18).

Normally, HGF in the lung is produced by cells of mesenchymal origin, mainly the stromal cells located in the submucosal areas just beneath the basement membrane of the epithelial cell layer (19). HGF is normally produced at low levels in the adult, but it is upregulated immediately following injury (20). Here we show that elevated production of HGF by airway epithelial cells increased incidence of lung tumor formation following exposure to a tobacco carcinogen. This important preclinical model of human lung adenocarcinoma will be useful to develop and test antitumor agents for lung cancer, especially those targeting HGF/c-Met signaling or downstream processes induced by phospho-c-Met such as angiogenesis or invasion. Additionally, this animal model may be useful for studying other diseases of the lung such as pulmonary fibrosis.

Materials and methods

Reagents

The CCSP antibody was a kind gift from Dr Susan D. Reynolds, University of Pittsburgh. HGF ELISA (enzyme-linked immunosorbent assay) kit, rHGF, goat polyclonal anti-HGF antibody and mouse monoclonal anti-HGF antibody were from R&D Systems (Minneapolis, MN). A549 lung tumor cells and Madin–Darby Canine Kidney (MDCK) cells were from the American Type Culture Collection (Manassas, VA). Nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was from Toronto Research Chemicals, North York, ON, Canada.

Construction of HGF transgene construct

A plasmid containing the HGF cDNA cloned into the pSPORT1 vector was a kind gift from Dr Reza Zarnegar at the University of Pittsburgh (14). For the promoter and downstream sequences, a plasmid containing the rat CCSP promoter cloned into the BamHI site of p0GH was obtained from Dr Brian Hackett at Washington University. The p0GH plasmid also contains the complete hGH gene (21). The 2.3 Kb CCSP promoter (~2332 to +40) and a fragment of the hGH genomic sequences were isolated from this plasmid and inserted into the pSPORT1 vector containing the HGF cDNA (Figure 1). A 1.6 Kb portion of the hGH polyadenylation site was included for proper processing of the mRNA to occur in transgenic mice and was inserted downstream of the 3' untranslated sequences of the HGF cDNA. The vector was sequenced and confirmed to be the correct sequence in the proper orientation at all junctions.

Generation and identification of HGF transgenic mice

A 5.9 kb human HGF transgene fragment containing the CCSP promoter, HGF cDNA and hGH polyadenylation site was excised from plasmid sequences by digestion with the Sphl restriction enzyme. The fragment was isolated from an agarose gel and purified using the Qiagen II Gel Extraction Kit (Qiagen, Valencia, CA) according to the manufacturers’ instructions. Microinjection of the fusion gene into the FVB/N1 mouse strain was performed by the University of Cincinnati Transgenic Mouse Facility.

Genomic DNA was isolated from mouse tail snips using the Easy-DNA Isolation Kit (Invitrogen, Carlsbad, CA) according to the manufacturers’ instructions. Samples were screened for the HGF transgene using PCR with the following conditions: 1X PCR buffer (Roche, Indianapolis IN), 2.5 mM MgCl2, (Roche), 1 µM HGF forward primer (5'-TGGAGGGAGCCAATAG-AAGGAG-3'), 1 µM HGF reverse primer (5'-GCCAGGCTGCGAGGATTTGG-3'), 0.5 mM PCR nucleotide mix (Roche), 200 ng heat-treated DNA and 2.5 U AmpliTaq Gold (Applied Biosystems, Foster City, CA). Positive control for HGF was uncut HGF transgene vector; negative control was water. Following PCR, products were separated on a 1% agarose gel and the expected size of HGF transgene product was 540 bp. All DNA samples were also screened for β-globin to ensure DNA integrity.

Isolation of conditioned medium and detection of secreted HGF

A549 cells were transiently transfected with the HGF transgene vector and Lipofectamine 2000 (Invitrogen) according to the manufacturers’ instructions. After 48 h, conditioned medium was isolated as described previously (22). From each sample, 25 µg of protein was separated on a 10–20% SDS–tricine–polyacrylamide gel and transferred to a nitrocellulose membrane. The membrane was immunoblotted with a 1:1000 dilution of monoclonal anti-human HGF antibody (specific reactivity to human HGF) followed by a 1:1000 dilution of a horseradish peroxidase-conjugated secondary antibody (Amersham, Piscataway, NJ). Immunoreactive bands were visualized by SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) followed by exposure to autoradiography film.

Scatter assay

MDCK cells were plated at a density of 1 × 10⁴ cells/well in 6-well plates. Tight colonies of cells were allowed to form for 3–5 days. Cells were serum-deprived overnight followed by addition of 50 ng/ml HGF, conditioned media from A549 transgene vector transfected cells, conditioned media from non-transfected A549 cells or no treatment. Amount of HGF in conditioned media from A549 cells was quantitated by densitometry and the amount of media that contained 50 ng/ml HGF was added to the wells. Cells were analyzed by microscopy 24 h after the addition of experimental treatments for spindle-shaped cells with pseudopodia-like protrusions, characteristics of scattered cells.

RNA isolation and northern analyses

RNA was isolated from tissues or tumors from transgenic and wild-type control animals by the guanidinium thiocyanate method (23). For tumors, animals were killed 40 weeks after a total of 24 mg NNK treatment to ensure presence of large tumors. Individual tumors were microdissected from the surrounding lung and placed in RNA later (Ambion, Austin, TX). Total RNA (10 µg/lane) was separated by size in a 1% denaturing agarose gel (0.66 M formaldehyde) and transferred to GeneScreen membrane (Perkin Elmer, Boston, MA) using a VacuGene XL blotting apparatus (Amersham). The transferred RNA was UV-cross-linked to the membrane by two cycles on a ‘autocrosslinker’ (120,000 µj/cm²) using a Stratallinker UV illuminator (Stratagene, La Jolla, CA) and hybridized with a random-prime labeled cDNA probe specific for the HGF transgene (Figure 1). Probes were generated using the DECAprime II random primed DNA labeling kit (Ambion) according to the manufacturers’ instructions. Hybridization and post-hybridization washes were as described previously (24,25). Radiographic signals on the washed filters were exposed to a phosphor screen for 4 days. Blots were stripped and reprobed with a GAPDH probe using DECAtemplate-GAPDH-mouse (Ambion).

Bronchioalveolar lavage and HGF quantification

A 22 gauge catheter was inserted into the trachea of killed mice and secured with surgical suture. Two 800 µl aliquots of 0.9% saline were serially instilled and withdrawn. The pooled sample was centrifuged at 3000 r.p.m. and the supernatant was removed and concentrated using a Microcon YM-3 centrifugal filter device (Millipore, Bedford, MA). The concentrated samples were then analyzed using the Quantikine HGF Immunoassay Kit according to manufacturer’s instructions. Ten transgenic and ten wild-type littermate control mice were used for the experiment. No cross-reactivity with mouse HGF was detected with either recombinant or endogenous mouse HGF in the ELISA assay (data not shown).

NNK carcinogenesis and histology

Mice were separated into groups by gender and HGF transgene status and given two i.p. injections of 3 mg NNK (15 µg/j) or 0.9% saline vehicle control over...
2 weeks. NNK is a potent mutagenic tobacco-specific carcinogen, which is known to induce lung tumors in mice. All experiments using HGF transgenic mice were performed using mice that were heterozygous for the transgene with high copy number. Mice were monitored weekly and killed at 10–38 weeks after the first injection. Lungs were inflated with 10% buffered formalin under 25 cm intra-alveolar pressure and removed. Tumors were counted under a dissecting microscope and measured using Motic Images 2000 software. Lungs were paraffin embedded and sections were stained with hematoxylin and eosin (H&E). All histological interpretation was performed under blinded experimental conditions by an expert lung pathologist. Animal care was in strict compliance with the institutional guidelines established by the University of Pittsburgh.

**Immunohistochemical staining**

Tissue samples were fixed in 10% buffered formalin. Tissues were paraffin embedded, sliced and mounted on slides. Paraffin was removed from the slides with xylenes, and slides were stained according to standard procedures. The HGF and CCSP double staining (Figure 5) was performed using a 1:10 000 dilution of rabbit polyclonal anti-rat CCSP antibody and a 1:10 dilution of goat polyclonal anti-human HGF antibody. The secondary antibodies were biotinylated IgG antibodies specific for the primary antibodies followed by horseradish peroxidase conjugated DAB/Ni chromogen (Vector Laboratories, Burlingame, CA) for HGF visualization and alkaline phosphatase conjugated New Fuschin chromogen (Vector) for CCSP visualization. Black staining was considered positive for HGF whereas red staining was considered positive for CCSP. Slides were counterstained with hematoxylin and analyzed using an upright Leica Microscope with attached digital camera. Single staining was performed for each antibody in addition to the double staining. Positive controls were placenta for HGF and rat lung for CCSP. Negative control staining was done without the addition of primary antibody.

Primary antibody used for HGF single staining (Figure 8) was a 1:10 dilution of goat polyclonal anti-human HGF antibody (specific reactivity with human HGF). The secondary antibody was a biotinylated IgG specific for the primary antibody. Brown staining was considered positive. The positive control was placenta and the negative control staining was done without the addition of primary antibody.

**Statistical analysis**

Poisson regression analysis was used for analyses of the number of tumors in the NNK carcinogenesis animal study. Logistic regression was used to test for differences in the numbers of mice with tumors. A repeated measures analysis was used for the tumor sizes in this experiment since there were multiple tumors from some of the mice. Significance tests were performed with two-sided significance level 0.05. The two sample Student’s t-test was used for analysis in Figure 4. All values are expressed as mean ± SD.

**Results**

**Construction of HGF transgene and verification of biological activities**

We have created the transgene vector as shown in Figure 1, in which 2.3 Kb of the human HGF gene is under control of the rat CCSP promoter. 1.6 Kb of the hGH 3′-untranslated sequences containing the polyadenylation signal was also included for proper processing of the transgene. The backbone vector is pSPORT1. The transgene was sequenced and verified to contain the correct sequences in the correct orientation. When transiently transfected into A549 lung tumor cells that normally do not express HGF, but will support transcription from the CCSP promoter because they express markers of type II pneumocytes (17), the vector was expressed and A549 cells secreted HGF protein (Figure 2A). A normal lung fibroblast (NLFB) cell line, which secretes HGF, was used as a positive control.

The biological activity of the protein produced by the transgene was also tested using a scatter assay. Scattering of MDCK cells by HGF is a well-documented assay to test the biological activity of HGF. Treatment of MDCK cells with conditioned media from A549 lung cancer cells transiently transfected with the transgene (Figure 2E) can induce cell scattering of MDCK cells similar to that observed with 50 ng/ml rHGF treatment (Figure 2C) compared with no treatment (Figure 2B). The conditioned medium from untransfected A549 cells cannot induce cell scattering (Figure 2D). These results confirm the ability of the transgene to be transcribed, secrete protein and exert biological effects in vitro in cells that are permissive for the CCSP promoter.

**HGF is expressed in the lungs of transgenic animals**

The transgene was sent to the University of Cincinnati Medical Center for production of transgenic mice in the FVB/N1 mouse strain, which is susceptible to lung tumorigenesis (26). Five founder mice were generated containing between 1 and 25 copies of the transgene based on genomic Southern analysis (data not shown). There were no apparent developmental or behavioral differences observed between transgenic and wild-type littermate control mice. In addition, there was no difference observed in pulmonary function between the transgenic and wild-type control mice (data not shown). Furthermore, the transgenic mice (up to 1 year of age) did not develop any dysplasia in the lungs versus wild-type controls although they showed evidence of a subtle Clara cell hyperplasia. The transgenic mice were fertile and produced normal offspring.

Northern analysis with a human HGF specific probe shows that in the HGF transgenic mice, the lung is the only tissue out of the panel of nine tissues examined that expresses the 2.3 Kb mRNA for the transgene (Figure 3). The endogenous mouse HGF mRNA is over 6 Kb and was not detectable in the northern analysis. This probe should detect mouse HGF as well as human HGF, if present. This reflects the low level of HGF mRNA expression under normal circumstances. No tissues from wild-type control mice expressed HGF transgene.
cells in the small airways of HGF mice versus wild-type mice was significantly increased by approximately 1.5-fold ($P = 0.0103$). Almost every cell in the bronchiole is expressing CCSP in the transgenic mice whereas only a portion of the cells are positive for CCSP in bronchioles from the wild-type mice. These results confirm that the transgenic mice secrete HGF protein in the airway lumen, as predicted by their genotype, and suggest that in the transgenic mice there is subtle Clara cell hyperplasia, possibly reflecting an autocrine response among cells that support transcription of CCSP.

Based on the expected permissibility of the CCSP promoter, most transgene expression should be located in the epithelial cells of the small airways. In order to verify which cell types in the lungs produce protein from the transgene, double immunostaining was performed to detect protein expression in the airway epithelial cells. In HGF transgenic mice, CCSP immunostaining (red precipitate, Figure 5A) co-localized with HGF immunostaining (black precipitate, Figure 5B) as shown by serial sections of the same airway. CCSP/HGF double immunostaining is shown in Figure 5C of representative small airways from a transgenic mouse. The dominant black precipitate, indicating HGF protein, is co-localized with the red precipitate indicating CCSP (visible as a tinge of red associated with the blackish/brown precipitate). Figure 5D shows the CCSP/HGF double staining from a wild-type mouse lung, demonstrating only CCSP staining (red color) in these airways with little or no HGF production (black color), as expected. These results confirm that the HGF transgene expression is found in the small airway Clara cells as expected in the transgenic animals only. Using an antibody that can detect both human and mouse HGF, the non-transgenic airway still showed almost no detectable staining (data not shown). c-Met expression was expressed at low levels uniformly in the normal Clara cells with no detectable difference between HGF transgenic and wild-type animals (data not shown).

**Distinct differences in mouse lung histology**

The transgenic mouse lung histology shows distinct alterations (Figure 6), although lung function in these animals is not impaired (data not shown). Based on H&E staining, the lungs from transgenic mice (4–5 months old) (Figure 6D–F) were congested in the alveolar spaces compared with wild-type controls (72 versus <5%). There was excess production of blood vessels in 38% of the transgenic animals compared with <5% of wild-type littermates (arrows, Figure 6E). There was also a convoluted pattern of airways that included wide bifurcations, and the airways did not have the normal tapering pattern compared with wild-type controls (Figure 6A–C). The ratio of airway width measured 125 μm above the branch point to 125 μm below the branch point was 2.36 ± 0.23 versus 1.26 ± 0.09 in wild-type ($n = 8$) versus transgenic animals ($n = 8$), respectively ($P < 0.0001$). This suggests that there was a problem with the development of branching, possibly because of unregulated HGF production. HGF is a known factor involved in branching in the developing lung (28).

**HGF transgenic mice are more susceptible to NNK induced lung carcinogenesis**

The HGF transgenic mice and wild-type littermate control mice were exposed to 6 mg of the tobacco carcinogen, NNK, which shows selective uptake into the airway epithelium and produces lung adenocarcinomas in mice (29). Based on the

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Fig. 3. HGF is only expressed in the lungs from transgenic mice. Total RNA was isolated from tissues from a transgenic mouse and analyzed using northern analysis with a probe specific for the HGF transgene. Blots were stripped and reprobed using a GAPDH probe.

Fig. 4. Transgenic mice express more HGF and CCSP. (A), Bronchioalveolar lavage was performed on 10 transgenic mice (HGF+) and 10 wild-type (WT) littermate controls and the levels of human HGF in the bronchioalveolar lavage fluid were measured by ELISA. Results are reported as the average ± SD. * $P < 0.0001$, two-tailed unpaired Student’s t-test. (B), Number of positive CCSP stained cells per 200 μm length of five bronchioles per slide (3 slides/group) were counted manually and the average ± SD was reported. ** $P = 0.0103$, two-tailed unpaired Student’s t-test.
Fig. 5. HGF is localized within the Clara cells. (A), Representative lung tissue section showing CCSP immunostaining (red color) of transgenic mouse bronchiole. (B), Representative lung tissue section showing HGF single immunostaining (black color) of transgenic mouse bronchiole. (C), Double staining of HGF (black) and CCSP (red) in transgenic lung bronchiole. Inset is a 1.5-fold high-powered view of double staining in epithelium. (D) double staining of HGF (black) and CCSP (red) in wild-type littermate control lung bronchiole. Scale bar: 50 μm.

Fig. 6. Effects of HGF transgenic expression on baseline lung histology. (A–C), H&E staining of sections of whole mouse lung preparations from representative wild-type littermate controls and (D–F), from 4-month-old HGF transgenic mice. Scale bars: 100 μm. Arrows indicated areas of increased blood vessel production.
combined data from H&E staining on serial sections and gross tumor visualization, the tumor number, size and histology were compared between the treatment groups. Figure 7 represents the combined data from two separate experiments and shows the number of mice per treatment group and time point. The white bars show the number of mice with no detectable tumors whereas the gray shaded bars represent the number of animals with at least one tumor. The number of mice with two tumors is the striped gray bar and the number of mice with three or more tumors is the striped black bar.

Statistical analysis demonstrates that the total number of tumors from NNK treated transgenic animals (mean 1.64, median 1, range 0–7, n = 44) versus the number of tumors from NNK treated non-transgenic animals (mean 0.70, median 0, range 0–4, n = 43) differs significantly (P = 0.0001, Poisson regression analysis). There was no difference in tumor number between males and females. The percentage of animals with at least one tumor was 75% (33/44) in the NNK treated transgenic group compared with 48.8% (21/43) in the NNK treated wild-type group and this was statistically significant (P = 0.013, logistic regression). The main effect was an increase in tumor multiplicity in HGF transgenic versus wild-type mice. At the later two time points, there was also considerable tumor formation with NNK in wild-type animals.

The size of tumors was also compared with repeated measures modeling analysis and the median or mean size was not significantly different between HGF transgenic and wild-type controls (P = 0.87). However, the range of tumor size in the transgenic group at 30 weeks was 0.148–6.654 mm² whereas the range in the wild-type group was 0.654–2.498 mm², indicating that at least some of the tumors grew larger in the transgenic animals. At 10 and 20 weeks, 100% of the tumors were papillary adenomas in both transgenic and wild-type animals. At 30 and 38 weeks, 75% of all tumors in HGF transgenic mice were adenocarcinomas and the remaining smaller tumors (25%) were adenomas. In wild-type mice, 62% of all tumors at 30 and 38 weeks were adenocarcinomas and 38% were adenomas.

We have also altered this protocol to decrease the time to tumor formation. Four times the amount of NNK was administered to the animals for a total dose of 24 mg NNK, the animals were killed 13 weeks after the first injection, and the number of tumors from NNK treated transgenic animals (mean 1.58, median 2, range 0–4, n = 12) versus NNK treated wild-type animals (mean 0.67, median 1, range 0–2, n = 12) was significantly higher (P = 0.04, Poisson regression analysis).

Tumors that arise in transgenic mice contain HGF protein
We have further shown that the lung tumors that arise in the transgenic mice contain high amounts of human HGF protein (Figure 8). Immunostaining with a human HGF specific antibody shows much increased HGF protein content in lung tumors arising from transgenic (Figure 8A and B) compared with wild-type mice (Figure 8C), suggesting that tumorigenesis is associated with functional transgene. All photographs were taken of areas on the section demonstrating the highest staining. Immunostaining with an antibody that detects mouse, rat and human HGF shows the same staining pattern (data not shown) indicating that the endogenous mouse HGF is barely detectable in these tumors. The HGF staining pattern in the tumors is a combination of membrane and cytoplasmatic staining suggesting internal production of HGF in at least some malignant cells. Additionally, a northern blot analysis of RNA isolated from tumors from transgenic animals show the 2.3 Kb HGF transgene RNA whereas tumors from wild-type animals do not (Figure 8D). This strongly suggests that in the transgenic animals, the tumors arose from airway epithelial cells that express the HGF transgene. It is also possible that some tumors arose from cells that bound HGF secreted by the transgene. This model may mimic lung tumorigenesis induced by a tobacco carcinogen that results in a tumor having a high HGF content. c-Met protein expression was observed in tumors from both transgenic and wild-type mice with uniformly low expression and no differences based on size of tumors (data not shown). CCSP immunostaining was negative in the tumors from both HGF transgenic and wild-type mice (data not shown). The lack of CCSP protein expression in lung tumors is consistent with other animal models that use the CCSP promoter for transgene expression. It appears that endogenous CCSP protein expression is down-modulated during the process of carcinogenesis (30). Some HGF protein expression in the tumors could also be arising from other areas of the lung, not necessarily only from within malignant cells in the tumor. Surfactant apoprotein C, an alveolar type II cell marker that may also show some expression in small airways, was also expressed in 81% of the tumors regardless of HGF genotype, tumor location or size of tumor. Another way to characterize whether the tumors arise from alveolar Type II cells or from Clara cells is to examine whether tumors show a solid or a papillary phenotype, respectively. In our cases, we observed 43% solid tumors in the pulmonary parenchyma and 57% papillary tumors associated with the airways. There was no difference in transgenic versus non-transgenic animals in this phenotype. Our results suggest that the tumors in this animal model may arise from both Clara cells and alveolar Type II cells, or from a cell that shows lineage with both, consistent with other reports (31,32).
Discussion

HGF transgenic mice with overexpression in the lung have been created and verified to be the expected genotype, and to show phenotypic evidence of HGF expression and expression of the c-Met protein in the airway allowing them to respond to HGF. Airway alterations were present in the transgenic animals, which included congestion, wide bifurcations and increased blood vessels. The main effect in tumor formation between transgenic and wild-type mice was an increase in tumor multiplicity; average tumor size was not different but the range of tumor size was much wider in the transgenic mice. Tumors arising after NNK exposure expressed c-Met and so have the capacity of responding to HGF. These results suggest that lung tumors induced by a tobacco carcinogen are promoted by expression of the HGF transgene, possibly through greater proliferation of individually mutated cells or by migration of tumor cells to other areas of the lung where additional tumors form. This is similar to a common type of clinical recurrence when a new tumor nodule arises, either in the ipsilateral or contralateral lung. The results also suggest that the increased NNK carcinogenicity is related to increased Clara cell numbers, since the Clara cell is believed to be a cell of origin for papillary adenocarcinoma.

Phenotypically, tumors arose in this animal model that had characteristics of both Clara cells and Type II pneumocytes. In this regard, two types of tumor phenotypes were present, papillary and solid, and some tumors expressed the alveolar Type II cell specific marker, SPC, whereas CCSP expression in most tumors was not detectable. These results are consistent with other transgenic mouse models utilizing the CCSP promoter (31,32). Since there are different lung adenocarcinoma features in human lung disease, including bronchioalveolar carcinoma, adenocarcinoma with bronchioalveolar features and pure adenocarcinoma, this animal model is representative of human lung adenocarcinoma disease.

Increased HGF and/or c-Met expression by human tumor cells have been associated with high tumor stage and poor prognosis in lung cancer. For example, in non-small-cell lung cancer patients, HGF is a useful prognostic indicator for both early and advanced disease (11). Additionally, in non-small-cell lung carcinoma cell lines and primary tumors, c-Met overexpression correlated with a higher tumor stage and worse outcome than those without overexpression (33). Coexpression of HGF and c-Met has been reported in approximately 50% of lung adenocarcinomas and this coexpression has been reported to correlate with poor prognosis (34,35). Furthermore, the survival rate for non-small-cell lung cancer patients with both intratumoral positive c-Met expression and stromal positive HGF expression was significantly lower than that for patients with only HGF or c-Met positive tumors as well as that for patients with both HGF and c-Met negative tumors (36). The strong correlation between HGF and c-Met expression and patient survival clearly supports the hypothesis that the HGF/c-Met pathway plays an important role in the pathogenesis of human cancers. These studies examined protein levels in tumors by immunoblotting or immunohistochemistry. Recently, HGF levels present in serum from breast cancer patients (37) and small-cell lung cancer patients (38) were measured by ELISA and were demonstrated to correlate with more advanced disease. HGF can potentially act as a serum tumor biomarker for different human cancers and possibly as a predictor for response to clinical therapy.

The results presented here are consistent with other animal models of HGF expression. In this regard, production of transgenic mice that express HGF under the control of promoters targeting gene expression to specific organs has resulted in tumor formation only in the organs where the genes are expressed. For example, transgenic mice have been produced that express HGF in the liver under the control of the albumin promoter (14) and these mice develop hepatocellular carcinomas. Transgenic mice have also been made that express HGF...
under the control of the metallothionein (MT) promoter. In this case, tissues where tumors were found are also those where the transgene expression is high including the mammary gland, muscle and skin (39) and diethylamino, an environmental carcinogen, has been shown to dramatically increase HGF-mediated tumorigenesis in the livers of MT–HGF transgenic mice versus wild-type controls (40). In another interesting model, MT–HGF transgenic mice in a scid background were demonstrated to have increased growth of subcutaneous xenografts from c-Met expressing cancer cell lines versus wild-type mice (41). Renal tubular hyperplasia has been reported in transgenic mice expressing HGF in the kidney (42). These results suggest that HGF can alter growth processes in many tissues and is an important oncogenic pathway in general. Overexpression in these tissues results in either spontaneous tumor formation or enhanced sensitivity to carcinogens. Recently, Frankel et al. (43) demonstrated that transgenic mice, over 12 months of age, that overexpress the human insulin-like growth factor-IA in the airways, show increased premalignant changes in the alveolar epithelium compared with wild-type littermate controls. Our model is the first animal model showing that growth factor modulation can increase lung cancer malignancies due to a tobacco carcinogen.

Because of the overwhelming evidence for the role of the HGF/c-Met pathway in the pathogenesis of human cancers, therapeutic inhibitors that target this pathway are being developed. A recent report described a small molecule inhibitor, PHA-665752, which shows >50-fold selectivity for the c-Met kinase compared with other tyrosine kinases and can inhibit the formation of phospho-c-Met in vitro and in vivo (44). Another small molecule inhibitor, SU11274, was reported to inhibit c-Met auto-phosphorylation and downstream signaling in non-small-cell lung cancer (45). However, these compounds have shown solubility problems and may not be feasible for clinical development. Recently, a single blocking anti-HGF antibody, L2G7, was shown to inhibit various biological activities induced by HGF in vitro and had profound antitumor effects on intracranial glioma xenografts (46). This single antibody approach is superior to other described neutralizing antibodies that target HGF but require a combination of at least three distinct antibodies binding to different epitopes for inhibition of HGF activity (47). Other potential inhibitors of this pathway include the HGF kringle variant antagonists such as the extensively studied NK4 (48), c-Met ribozyme (49,50), c-Met antisense (51), c-Met dominant negative (52) and decoy receptors (53) and c-Met peptide antagonists (53,54). Collectively, these studies show that the HGF/c-Met pathway is an attractive target for therapy and could be clinically useful for many human cancers, especially lung.

These experiments have demonstrated the ability of upregulated HGF expression in the lungs to promote tumorigenesis and tumor progression, and have corroborated our observations that lung cancer patients with elevated HGF have more aggressive disease (11). Although we did not find an increase in the rate of spontaneous tumor formation in this model, the differences observed in the carcinogen-induced model were significant. This animal model recapitulates the phenotype of aggressive lung adenocarcinoma that overexpresses HGF and will be useful to test inhibitors of the HGF/c-Met signaling pathway as well as other novel therapeutic inhibitors for lung cancer such as those targeting angiogenesis, invasion or cell migration. We have recently determined that the HGF/c-Met pathway can activate the enzyme cyclooxygenase 2 (COX-2) via the MAP kinase pathway in non-small-cell lung cancer cell lines in vitro, which can result in upregulation of important pathways that are controlled by prostaglandins. Thus, this animal model can also be used to test inhibitors of the COX-2 pathway alone and in combination with HGF targeted inhibitors.

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References


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