Constitutive Achaete-Scute Homologue-1 Promotes Airway Dysplasia and Lung Neuroendocrine Tumors in Transgenic Mice

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Abstract

The transcription factor achaete-Scute homologue-1 (ASH1) is essential for neural differentiation during fetal development and is a cardinal feature of neuroendocrine (NE) tumors such as small cell lung cancer. To explore the potential of ASH1 to promote NE differentiation and tumorigenesis in the lung, we constitutively expressed the factor in nonendocrine airway epithelial cells using transgenic mice. Progressive airway hyperplasia and metaplasia developed beginning at 3 weeks of life. ASH1 potently enhanced the tumorigenic effect of SV40 large T antigen in airway epithelium. These doubly transgenic animals developed massive NE lung tumors, implying that ASH1 may cooperate with defects in p53, pRb, or related pathways in promoting NE lung carcinogenesis.

Introduction

A striking feature of SCLC, as opposed to other major forms of lung cancer, is the presence of neural and NE differentiation features. Certain NE features may contribute to SCLC virulence. Autocrine loops involving gastrin-releasing peptide and other neuropeptides, along with their cognate receptors, promote cell growth and protect against apoptosis in SCLC cell lines in culture (1). In addition to classic SCLC and less malignant bronchial carcinoid tumors, a subset of non-SCLC tumors, comprising approximately 8% of human lung cancers, exhibit NE features (2). A useful approach to understanding mechanisms regulating NE differentiation in normal lung and lung cancer has been to study transcription factors operating in fetal nervous system development. Our earlier studies suggest that ASH1 (also termed MASH1), a basic helix-loop-helix transcription factor conserved from the Drosophila achaete-scute complex, plays a critical role in regulating the NE phenotype in normal lung and in lung cancer (3). For example, transgenic knockout of the transcription factor results in a complete failure of NE cells to develop in the lung (3). In addition, these MASH1Δ animals are known to have defects in elaboration of central nervous system; in autonomic, enteric, olfactory, and retinal neurons; and in thyroid calcitonin-producing C cells (4–7). In the nervous system, ASH1 expression appears largely restricted to mitotically active precursor cells and is silenced before terminal differentiation. Interestingly, MASH1Δ mice exhibit a hypoproliferative phenotype in the cerebral cortex (8). In the context of the lung, ASH1 is specifically expressed in pulmonary NE cells and in lung cancer cells with NE features (3). However, it is presently unknown whether ASH1 is sufficient to confer NE differentiation on lung cancer cells or on their normal airway epithelial counterparts. Similarly, the potential of the transcription factor to influence lung cell proliferation and tumorigenesis is unknown. We therefore created a transgenic mouse model to constitutively express ASH1 in nonendocrine airway epithelial cells that normally lack this factor. Remarkably, we find that ASH1 can promote airway epithelial proliferation and can dramatically potentiate the tumorigenic impact of loss of p53 and pRb function, resulting in lung cancers resembling human non-SCLC NE carcinoma.

Materials and Methods

Generation of CC10-hASH Transgenic Mice. The mouse CC10-hASH transgene was cloned by fusing 1.1 kb of sequence containing the coding region of the hASH1 gene from pM1.1 (9) with 2.1 kb of upstream 5′-flanking sequences of the mCC10 promoter (10). Briefly, an EcoRI fragment of the hASH1 gene was cloned into the EcoRI site in a mCC10 expression vector consisting of the HindIII- Hph1 fragment of the mCC10 promoter cloned into the BamHI site of pBSK(–). Cloned into this plasmid were rabbit globin gene intron sequences (into a BamHI site to EcoRI site) and the bovine growth hormone polyadenylation signal (XbaI to XhoI fragment from pGKneo cloned into the EcoRV site). A BsrX1-KpnI fragment containing the transgene was gel-purified using Quex resin and eluted into 60 μl of modified Tris-EDTA. The transgene was microinjected into one-cell fertilized B6D2F1 × ICR mouse embryos at a concentration of 2 ng/μl as described previously (11). Implantation into pseudopregnant females was performed as described previously (12). Transgene incorporation was verified using tail DNA Southern analysis using a transgene probe as well as transgene-specific PCR. All animals were housed and handled in a humane manner in an AAALAC-accredited facility in accordance with the standards set forth by the NIH Guide.

Lung RNA Isolation. After avertin anesthesia, lungs were removed and frozen in aluminum foil with dry ice. Mouse lung tissue for RNA was homogenized in 5 ml of Trizol reagent (Life Technologies, Inc.) by six 10-s bursts at a setting of speed 5. One ml of chloroform was added to the homogenized tissue, shaken vigorously, and then centrifuged at 12,000 rpm in bursts at a setting of speed 5. One ml of chloroform was added to the homogenized tissue, shaken vigorously, and then centrifuged at 12,000 rpm in 15 min, washed with 70% ethanol, resuspended in diethyl pyrocarbonate H2O, and analyzed by spectrophotometry at 260 and 280 nm.

RPA. hASH1 cDNA templates (9) for RPA were linearized by digestion with NcoI. To generate the riboprobe, 1 μl of template was subjected to in vitro transcription in a 20-μl reaction containing 1× transcription buffer; 10 mM DTT; 20 units of RNasin; 0.5 mM each of ATP, CTP, and GTP; 50 μCi of [α-35S]UTP; and 15–20 units of T7 RNA polymerase. The reaction was incubated at 37°C for 1 h, 2 units of DNase I (Ambion, Austin, TX) were added, and the reaction was terminated 30 min later with 5 μl of 0.5 M EDTA. After extraction with phenol/chloroform/isomyl alcohol (25:24:1 pH 5.2), the aqueous phase was purified on a Sephadex G50 column. The eluted solution was diluted 1:10 and counted using a scintillation counter. RPA were performed using the standard protocol as supplied by Ambion. Briefly, total RNA (5 μg) was combined with specific riboprobe (ranging from 5 × 105 to 5 × 106 cpm) and hybridized for 18 h at 65°C. After hybridization, the RPA blots were washed and exposed to X-ray film.
was performed as described previously (3, 14). Immunostaining for hASH1 (1:500), CGRP (1:3,000; Amer-...d in the epithelium of the distal airway, the Clara cells (15). Clara cells, or their immediate progenitors, may help to reconstitute several airway epithelial cell populations after lung injury (16). Persistent expression of genes under the control of the CC10 promoter begins at approximately embryonic day 16 in developing mice, continuing throughout postnatal life (13). We used this well-characterized transgenic mouse system to investigate how ASH1 may influence the differentiation of airway epithelial cell precursors. Preliminary data suggested that the CC10 promoter would direct heterologous expression of the CC10-hASH1 transgene to non-NE lung cells that do not normally express ASH1. In normal mice, CC10-reactive airway epithelial cells are typically negative for immunoreactive ASH1 (data not shown). In addition, the MASH1Δ transgenic knockout strain exhibits a normal expression pattern for CC10, despite the absence of ASH1 and lung NE cells (3). We subcloned a hASH1 cDNA (9) containing the complete coding sequence downstream of the 2.1-kb murine CC10 promoter, injected oocytes, and implanted the oocytes in pseudopregnant females. Founder lines were characterized by Southern analysis and PCR of tail DNA. Two founder lines, 1229 and 1230, were subsequently found to have both genomic incorporation of the human transgene and detectable expression by RPA of whole lung total RNA (see Fig. 1, Lanes 6–9). These two founder lines, with normal viability and fertility, were used for subsequent studies.

The lung phenotype of CC10-hASH1 transgenic mice was striking. Beginning as early as 3 weeks after birth, we observed epithelial cell hyperplasia in the distal airways, concentrated at the bronchioalveolar junction. There was also progressive bronchiolarization of the alveoli, with metaplasia of proximal alveolar ducts by bronchiolar epithelium composed of ciliated and CC10-reactive cells (Fig. 2B, C, and E). In addition, bronchioli exhibited a loss of the characteristic hobnail appearance of nonciliated cuboidal epithelia. After 8 weeks, there was redundant folding of the epithelial surface in a papillary configuration in addition to increasing hyperplasia and metaplasia at the bronchioalveolar junction. At 9 months of age, extensive hyperplasia and metaplasia at the bronchioalveolar junction was accompanied by foci of lepidic, glandular, and papillary growth in the alveolar compartment (Fig. 3, A and B). We confirmed that the majority of these hyperplastic epithelial cells were immunoreactive for both hASH1 and CC10 (see Fig. 2, D and E). A similar response was seen for both the 1229 and 1230 founder lines.

A critical question in these studies was whether ASH1 would be sufficient to induce the expression of NE differentiation markers in these heterologous airway epithelial cells. Although CC10 and NE markers may be transiently coexpressed in the early undifferentiated lung bud (17), these markers identify distinct cell populations in late prenatal and postnatal lung. It is presently unclear whether Clara cells and lung NE cells may in fact derive from a common precursor pool. In these CC10-hASH1 transgenic animals, we observed a transient increase from 0.26 to 0.46 (77%) in the number of CGRP-containing NE cell foci per airway, composed of either isolated NE cells or small clusters, compared with wild-type littermates at 3 weeks of age. However, cells contributing to distal airway hypertrophy and bronchiolar metaplasia were consistently negative for NE marker reactivity (Fig. 2F). Therefore, it appears that constitutive hASH1 overexpression can lead to a significant hyperplastic response and cellular metaplasia but not to overt NE differentiation in the context of the normal distal airway epithelial cells that use the CC10 promoter. Conceivably, at the point at which the CC10 promoter has become active, host cells may be committed to an airway epithelial cell lineage to a degree that they are incapable of responding to hASH1 with NE differentiation. Alternatively, dimerization partners of hASH1, such as E12 and E47 or other necessary transcription factors, could be present in limiting amounts in these cells.
Cooperation Between hASH1 and SV40 Large T Antigen in Lung Tumorigenesis. In prior studies, the SV40 large T antigen, under the control of the CC10 promoter, was shown to be a potent inducer of lung adenocarcinomas, with microscopic tumors appearing as early as 1 month of life (12). Principal actions of the oncprotein in this setting include sequestration of Rb and p53, mimicking important molecular alterations seen in human lung cancer. In addition, SV40 large T antigen potentially can modify the function of CREB binding protein/p300, affecting many differentiation processes within the cell (18). Based on the impressive hyperplastic response to the CC10-hASH1 transgene, we sought to determine whether hASH1 could cooperate with the proliferative and tumorigenic effects of large T antigen. Therefore, we crossed CC10-hASH1 mice with an existing strain of CC10-SV40Tag mice, 7736 (12). At each time point tested, beginning with newborn pups, doubly transgenic mice exhibited substantial airway hyperplasia and dysplasia that could readily be distinguished from the milder phenotypes seen in either singly transgenic strain. This difference was obvious by 3 weeks of life, when the doubly transgenic animals exhibited marked generalized airway epithelial dysplasia and focal hyperplasia, and adenoma formation centered around distal airways (see Fig. 3, C and D). Dysplastic growth frequently obliterated the bronchioalveolar junctions. By 2–4 months of life, doubly transgenic mice had extensive lung replacement by solid adenocarcinomas (see Fig. 3, E and F). These tumors were far more extensive than those seen in the CC10-SV40Tag mice, which were consistently less than 0.5 mm in diameter at this time point (see Fig. 3, G and H). Increased hASH1 expression was verified by immunohistochemistry and by RPA (Fig. 1A, Lanes 10–14). The high level expression of hASH1 in doubly transgenic lungs compared with that in CC10-hASH1 lungs (Lanes 6–9) appears to be related, in part, to the higher fraction of lung cells expressing the transgene in these tumor-laden doubly transgenic lungs. The accelerated airway epithelial hyperplasia and formation of aggressive cancers described above imply a synergistic interaction between hASH1 and SV40 large T antigen in airway cell tumorigenesis.

NE Differentiation in Hyperplastic Airways and Lung Cancers Induced by hASH1 and SV40 Large T Antigen. Beginning at 6 days of life, doubly transgenic animals were observed to have generalized distal airway epithelial dysplasia and hyperplasia, but no increase was seen in the relative numbers of NE cell foci. Remarkably, by 2–4 months, a high percentage of these proliferative epithelial cells exhibited immunoreactivity for the NE markers synaptophysin and CGRP (Fig. 4B–D). Such diffuse NE marker reactivity was never observed in either the CC10-hASH1 or CC10-SV40Tag strains alone (see Fig. 4A for comparison). NE reactivity was confined to distal airway epithelial cells, correlating with transgene expression and focal immunoreactivity for CC10. The degree of this generalized NE transdifferentiation was positively correlated with the size of developing tumors in adjacent lung. Moreover, the resulting adenocarcinomas exhibited frequent NE differentiation as well. By immunohistochemistry, tumors were positive for synaptophysin, PGP9.5, CGRP, and, to a lesser degree, chromogranin, as well as CC10 (see Fig. 4, E and F; data not shown). Altogether, 16 of 18 lungs from doubly transgenic animals had NE-positive tumors, and 14 of these lungs had focal NE reactivity in tumors, whereas 2 had diffuse tumoral expression of these markers. In contrast, at no point did tumors found in singly transgenic CC10-SV40Tag mice express any of these NE markers. In summary, tumors emerging from the doubly transgenic mouse background striking resemblance to the non-SCLC with NE features that spontaneously arise in human tobacco smokers (2, 13).
Discussion

Our studies reveal several unexpected properties of the neural developmental transcription factor ASH1. Not only can this factor promote NE differentiation in developing airway epithelial cells normally lacking NE properties, but the hASH1 transgene also magnifies the oncogenic potential of SV40 large T antigen, contributing to the emergence of aggressive adenocarcinomas with NE features. Although no naturally occurring genetic amplification, rearrangement, or activating mutations of hASH1 in tumors have been observed to date, high-level expression of hASH1 is uniformly present in classic SCLC, non-SCLC NE, and bronchial carcinoid tumors and cell lines (3). In the current studies, this transcription factor displays some of the hallmarks of a cellular proto-oncogene, whereby constitutive overexpression can potentiate the tumorigenic effects of a bona fide viral oncogene.

In the absence of SV40 large T antigen, hASH1 promotes airway epithelial cell hyperplasia and metaplasia, with bronchiolarization of proximal alveolar spaces. Although this phenotype did not produce frank carcinomas or large adenomas, airway cells expressing the transgene exhibited progressive growth throughout the life of the animals. Because ASH1 is not expressed in terminally differentiated adult neuronal cells and is markedly down-regulated in adult lung as well, it is conceivable that constitutive expression of this factor may not be compatible with end-stage differentiation of airway epithelial cells. Interestingly, the vast majority of hyperplastic and metaplastic airway epithelial cells remained negative for NE differentiation markers in the absence of large T antigen. This requirement for further disruption of epithelial differentiation by T antigen suggests that most airway cells are sufficiently committed to an epithelial lineage such that hASH1 alone cannot induce overt NE trans-differentiation. We suspect that the developmental plasticity of airway epithelial precursors using the CC10 promoter may contribute to the proliferation of hASH1-expressing cells in this model system. To further clarify the association of the CC10 promoter with multipotential airway epithelial progenitors, it would be instructive to overexpress hASH1 under the control of other lung-related promoters.

In the context of coexpression of SV40 large T antigen under the control of the CC10 promoter, hASH1 promotes both the expression of NE phenotype in airway epithelial cells and the emergence of aggressive NE lung carcinomas. Each of the known functions of the multifunctional SV40 large T oncoprotein appear to be relevant to

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Fig. 3. Advanced tumorigenesis in doubly transgenic animal lungs. A, a whole lobe cross section from CC10-hASH1 lung at 9 months shows minute foci of aberrant growth mainly at bronchiole/veolar junctions. B, high power view from the same lung shows extensive bronchiole/veolar metaplasia (arrow; H&E stain; ×70). C, a whole lobe cross-section from CC10-hASH1-TAg lung at 3 weeks reveals multiple tiny adenomas, many of which are located adjacent to airways. D, high power view from the same lung shows extensive dysplasia of the airway epithelium that extends to the alveolar compartment (arrow; H&E stain; ×70). E, extensive tumors have replaced the entire lobe of CC10-hASH1-TAg lung at 3 months. F, high power view confirms that the tumors are adenocarcinomas, frequently encasing the airways (H&E stain; ×70). G, in contrast, a whole lobe cross-section from CC10-TAg lung at 3 months reveals multiple small adenomas, less than 0.5 mm in diameter, distributed mostly around small airways and bronchiole/veolar junctions. H, a corresponding high power view shows airway cell hyperplasia with severe dysplasia and an adenoma (arrow; H&E stain; ×70). Bar, 2 mm. Lu, bronchiolar lumen.

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Fig. 4. Extensive NE differentiation in the airway epithelium and tumors of doubly transgenic animals (immunoperoxidase staining; ×150). A, for comparison, scattered NE cells (arrow) and nerves (arrowhead) containing synaptophysin in the airways of CC10-hASH1 mice are shown. In contrast, serial sections of CC10-hASH1-TAg bronchus reveal marked hyperplasia of NE cells (arrow) containing (B) PGP9.5, (C) synaptophysin, and (D) CGRP immunoreactivity. E, synaptophysin immunoreactivity in a CC10-hASH1-TAg pulmonary adenocarcinoma that also expresses (F) CC10. Lu, bronchiolar lumen; T, tumor.

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R. I. Linnoila, unpublished data.
SCLC biology and may be important for synergy with hASH1. Both pRB and p53 are nearly universally inactivated in SCLC, which characteristically expresses hASH1. The status of the CREB binding protein/p300 transcriptional coactivator in SCLC is more difficult to determine. Although transcriptionally active complexes involving cAMP-responsive elements and coactivators appear to exist in cultured SCLC, it is unclear whether p300 function is modified in this cancer (19).

The doubly transgenic CC10-hASH1-TAg tumor model reported herein appears to be a unique, reproducible, and potentially valuable animal model for NE lung tumors. To date, available rodent models have not faithfully recapitulated NE lung cancer, especially the distinctive features of human SCLC. Overexpression of Ha-ras using the calcitonin-CGRP promoter induces pulmonary NE cell hyperplasia and predominantly non-NE tumors as well as aggressive medullary thyroid cancers (20). Whereas the exposure of hamsters to nitrosamines also leads to pulmonary NE hyperplasia, in most cases, the resulting lung tumors lack a NE phenotype (21). The new model system reported in our study is particularly attractive in the sense that it uses molecular alterations seen in native lung cancer and results in predominantly NE lung tumors. From a tumor biology perspective, this doubly transgenic tumor model affords the opportunity to characterize critical mediators of NE features and their relationship to tumor virulence in the course of tumor evolution. From a treatment-oriented perspective, this model provides a novel and potentially valuable system for evaluating prophylactic, differentiation-based, gene transfer, and cytotoxic therapy for NE lung carcinomas.

References
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