Oxygen Concentration Determines the Biological Effects of NOTCH-1 Signaling in Adenocarcinoma of the Lung

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Abstract

NOTCH signaling is an evolutionarily conserved signaling pathway that regulates cell fate during development and postnatal life. It has been increasingly linked to carcinogenesis, although its role in cancer seems to be highly context and tissue specific. Although NOTCH signaling is required for lung development, little is known about its role in lung cancer. In this study, we show that NOTCH signaling, as measured by the γ-secretase cleavage product NIC-1, is active in both normal human and lung tumor samples; however, downstream NOTCH readouts (i.e., HES-1 and HES-5) are elevated in lung tumors. Levels of NOTCH signaling components in primary human lung cells reflect observations in tissue samples, yet lung tumor cell lines showed little NOTCH signaling. Because oxygen concentrations are important in normal lung physiology and lung tumors are hypoxic, the effect of low oxygen on these lung tumor cell lines was evaluated. We found that hypoxia dramatically elevates NOTCH signaling (especially NOTCH-1) in lung tumor cell lines and concomitantly sensitizes them to inhibition via small-molecule γ-secretase inhibitors or NOTCH-1 RNA interference. γ-Secretase inhibitor–induced apoptosis of lung tumor cells grown under hypoxic conditions could be rescued by reintroduction of active NOTCH-1. Our data strengthen the role of NOTCH in lung cancer and as a therapeutic target for the treatment of lung and other hypoxic tumor types. [Cancer Res 2007;67(17):7954–9]

Introduction

Lung cancers are the most common malignancies in the United States, accounting for 31% of male and 26% of female cancer-related deaths (1). Adenocarcinoma of the lung (ACL) represents ~50% of all lung cancers (2), and 15% to 20% of ACL cases occur in nonsmokers (2). Genetic data in nonsmokers (2) suggest that unidentified carcinogens may be one cause of ACL in the nonsmoking population. Despite treatment, the 5-year survival rate for ACL is 15% (1); thus, it is imperative that novel targets and therapies are identified.

NOTCH signaling is an evolutionarily conserved pathway that regulates critical cell fate decisions (3). In humans, NOTCH signaling is mediated by a family of transmembrane receptors (NOTCH-1 to NOTCH-4) and ligands (JAGGED-1 and JAGGED-2 and DELTA-like 1, 3, and 4; ref. 3). NOTCH receptors consist of a modular NH2-terminal extracellular subunit (NRC) noncovalently bound to the COOH-terminal transmembrane domain (NTM) subunit (4). NOTCH ligands are single-pass transmembrane proteins that are presented to NOTCH receptors by a neighboring cell. On ligand binding, NOTCH receptors undergo proteolytic modifications in the NTM, which makes them susceptible to final cleavage by a presenilin-1–dependent γ-secretase (3). This process leads to the release of the activated form of NOTCH (intracellular NOTCH or NIC), which translocates to the nucleus where it modulates gene expression primarily by binding to ubiquitous transcription factor CBF-1 in humans (3). NOTCH target genes include several helix-loop-helix transcription factors collectively named Hairy/enhancer of split (HES) and HEY (3). Many of these are negative transcriptional regulators that inhibit differentiation-inducing factors during development contributing to the maintenance of a precommitted cell state for proper interpretation of differentiation or proliferation stimuli (3). Knockout mouse studies have shown that NOTCH signaling is required for lung development (5). During postnatal life, NOTCH regulation of cell proliferation and apoptosis is context dependent (6) and although in certain tissues NOTCH is suggested to play a tumor suppressor role (6), NOTCH signaling is increasingly linked to oncogenicity (7). In light of mounting evidence for a role of NOTCH in cancer, little is known about NOTCH in lung cancer. Although expression of constitutively active NOTCH-1 caused growth arrest in small cell lung cancer cells (8), NOTCH-3 seems overexpressed in 30% to 40% of nonsmall cell lung cancer (NSCLC; ref. 9). Moreover, a t(15:19) chromosomal translocation has been detected in some lung cancer and derived cell lines, suggesting that NOTCH-3 may be an oncogene in NSCLC (10). Here, we have studied the expression levels and the biological effects of NOTCH signaling in ACL using cell lines and frozen tumor biopsies.

Materials and Methods

Cell culture, hypoxia, and γ-secretase inhibitor. Human bronchial epithelial cells 4F0439 and 4F0624 and small airway epithelial cells 3F1584, 4F0001, and 4F0715 were cultured as recommended (Cambrex). Human lung fibroblasts MRC5 and CRL-7285 [American Type Culture Collection (ATCC)] were cultured in DMEM with 10% fetal bovine serum (FBS). We used NSCLC cell lines of different histologic subtypes [i.e., H226 and HCC95 (squamous cell carcinomas); HCC1171 (large cell carcinoma); H1395, H1755, HCC2374, A549, HCC827, H1299, and H2347 (adenocarcinomas)]. All lines were from ATCC. Cancer cell lines were grown in RPMI 1640 with 10% FBS. All cells were fingerprinted using the GenePrint fluorescent STR system (Promega). Cells grown in hypoxia were maintained in chambers...
(Stem Cell Technologies) filled with certified 1% O₂, 5% CO₂, and 94% N₂ (Airgas North Central) at 37°C. Oxygen concentration was measured with MiniOXI oxygen meters (Mine Safety Appliances Co.). We used the γ-secretase inhibitor MK0003 (11). This compound was dissolved in DMSO to make 40 mmol/L stock solutions.

Plasmids and lentiviral vectors. The pN²²–¹ plasmid expresses NOTCH-1 N²² cloned into the BamHI and EcoRI sites of pcDNA3.0 (Invitrogen; ref. 12). This same cDNA was inserted into pLenti4/TO/V5-DEST (tetracycline-inducible ViraPower T-Rex Lentiviral system, Invitrogen) to obtain pN²²–¹-DEST plasmid. pN²²–¹ and control vectors were transfected by electroporation. The vector expressing a short hairpin targeting NOTCH-1 (shN1) was constructed by annealing two oligonucleotides (5′-GATCTCTGGAGCCACGCGCTGCTGATCAGATCAATGGTGGA-AAGCATGATTCTGATGACTGCACAGGTGAACTGCTGGACGACGCGCTGCTGCCCTCGAG-3′ and 5′-AATTCTCTGAGGACGCGCTGCTGGACAAAGATCATTGCATCTGCTTCCACATTTGAGATCTTGGATCCAGGCAGCGGTCGCTCTCGAG-3′). The resulting dsDNA was ligated into the BamHI and EcoRI sites of pENTR-R-Gus (Invitrogen). The shN1 sequence was transferred to lentiviral expression vector pLenti4/TO/V5-DEST to generate pshN1-DEST following the manufacturer’s instructions. To generate tetracycline-inducible, stable cell lines, AC1 cells were first infected with tetracycline regulator lentivirus. Stable tetracycline regulator–expressing cells were then infected with pN²²–¹-DEST virus, pshN1-DEST virus, or pDEST virus, respectively. Lentiviral packaging and selection of transduced cells were done as recommended (Invitrogen). Doxycycline-inducible expression of N²²–¹ was verified by Western blot.

Antibodies. We used the following antibodies: rabbit polyclonal anti-NOTCH-1 (C-20), rabbit polyclonal anti-NOTCH-3 (M134), rabbit polyclonal anti-NOTCH-4 (H-225), goat polyclonal anti-JAGGED-1 (C-20), mouse polyclonal anti–hypoxygen-inducible factor (HIF-1α; 28b), mouse monoclonal anti-p53 (DO-1)-horseradish peroxidase, goat polyclonal anti-AKT (C20), rabbit polyclonal anti-BAX (N-20), and mouse monoclonal anti-BCL-2 (C-2; all from Santa Cruz Biotechnology). Rabbit polyclonal anti-NOTCH-2 was from Abcam. Rabbit polyclonal anti-cleaved NOTCH-1 (Val1745), rabbit monoclonal anti–phospho-AKT (Ser473), rabbit polyclonal anti–c-Jun NH²-terminal kinase (JNK), and rabbit monoclonal anti–phospho-JNK (T183/Y185) were from Cell Signaling. Mouse monoclonal anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Chemicon. One hundred micrograms of total cell lysates were run onto 10% SDS-PAGE and assayed by Western blot following standard procedures. Immunohistochemistry on frozen biopsies was done following standard procedures.

Real-time reverse transcription-PCR. Total RNA from cultured cells was extracted with RNeasy Mini kit, whereas RNA from frozen biopsies was extracted with RNeasy Micro kit (Qiagen). cDNA was synthesized with First-Strand cDNA synthesis kit (Fermentas). Quantitative real-time PCR was done with SYBR Green PCR Master Mix (Applied Biosystems) in an ABI 7300 thermal cycler (Applied Biosystems). Primer sequences are listed in Supplementary Table S1. For each sample, a serial dilution of cDNA template was measured in triplicate. Non–reverse transcription reactions served as controls. All measurements were normalized for 18S rRNA. Comparison between groups were analyzed by Student’s t-test, with α = 0.05.

Cell viability assays. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cytotoxicity assay kit (Roche). The results were verified by trypan blue assays. Results were expressed as mean ± SD of three independent experiments. Comparison between control and γ-secretase inhibitor treatment was analyzed by Student’s t-test. Apoptosis was measured by Annexin V-phycocyanin/7-aminoactinomycin D (7-AAD) fluorescence-activated cell sorting (FACS; BD FACSCanto; Becton Dickinson).

Figure 1. NOTCH signaling pathway is down-regulated in NSCLC cell lines compared with normal airway epithelial cells. A, Western blot analysis of NOTCH-1 expression levels in primary airway epithelial cultures (AEC), lung fibroblasts (Fibr.), and NSCLC cell lines. GAPDH was used as a load control. B, expression of NOTCH ligands and other NOTCH receptors in cultured cells analyzed by Western blot. C, expression of NOTCH-1 mRNA in cultured cells measured by real-time reverse transcription (RT-PCR). D, expression of HES-5 mRNA in cultured cells. In (C) and (D), expression levels of the genes of interest were normalized to 18S rRNA as described in Materials and Methods with expression set relative to BE1 (100 arbitrary units). Columns, relative expression (six individual measurements); bars, SD. BE, bronchoepithelial cell (BE1, 4F0439; BE2, 4F0624); SA, small airway epithelial cell (SA1, 4F0001; SA2, 4F00715; SA3, 3F1584); FB, fibroblast (FB1, MRC5; FB2, CRL-7285); SC, squamous cell carcinoma cell line (SC1, H226; SC2, HCC95); LC, large cell carcinoma cell line (LC1, HCC2171); AD, adenocarcinoma cell line (AD1, H1395; AD2, H1755; AD3, HCC2374; AD4, A549; AD5, HCC827; AD6, H1299; AD7, H2347).
Results and Discussion

We measured the expression levels of the four NOTCH receptors and ligands JAGGED-1 and DELTA-1 and DELTA-4 in 10 NSCLC cell lines compared with primary bronchoepithelial, small airway epithelial, and lung fibroblast by Western blot (Fig. 1A and B). All NSCLCs showed reduced expression of several components of the NOTCH signaling pathway compared with primary bronchoepithelial and small airway epithelial. The seven ACL lines displayed reduced or undetectable amounts of NOTCH-1 (Fig. 1A), with similar observations made at the mRNA level (Fig. 1C). The mRNA expression levels of NOTCH downstream effector HES-5 reflected what was observed at the protein level with exception of two NSCLC lines (Fig. 1D). Similar results were obtained measuring other NOTCH targets, such as HES-1 and HEY-1 (data not shown). Overall, the expression levels of NOTCH components in ACL cell lines were more similar to those of lung fibroblasts than to those of primary lung epithelial cells, suggesting epithelial to mesenchymal transition. Because primary cells and cell lines are cultured in different media, we tested whether this could explain differences in expression. We compared NOTCH-1 expression levels in one ACL cell line and in one SA culture grown in RPMI 1640 and the media used for primary cells. We detected no differences in NOTCH-1 levels (Supplementary Fig. S1). Because NOTCH-1 expression seemed to be specifically lost in ACL cell lines, we focused on this receptor. We tested 11 matched frozen biopsies of ACL and normal lung by immunohistochemistry using a highly specific antibody recognizing NIC-1 (active NOTCH-1; Supplementary Fig. S2). Lung epithelia showed a strong nuclear staining for N\textsuperscript{IC-1}, whereas ACL samples displayed reduced N\textsuperscript{IC-1} expression, with some tumor areas showing undetectable staining (Supplementary Fig. S2B and C).

When we expressed N\textsuperscript{IC-1} in ACL lines either through transient transfection or via a doxycycline-inducible lentiviral vector, N\textsuperscript{IC-1} induced apoptosis (Fig. 2) mediated at least in part by reduced expression of antiapoptotic proteins BCL-2 and MCL-1 and by increased expression of tumor necrosis factor–related apoptosis-inducing ligand (Supplementary Fig. S3). These results suggested that NOTCH-1 could play a tumor-suppressive role in ACL. We therefore tested the hypothesis that NOTCH signaling is reduced in ACL compared with normal lung by measuring HES-1 mRNA expression in matched tumor lung biopsies. The results showed that mRNA expression of this NOTCH downstream transcription factor was either the same or increased in ACL biopsies compared with normal lung (Supplementary Fig. S4). Similar results were obtained when measuring the HES-5 mRNA expression levels (data not shown). This suggested that the activation status of the pathway does not correlate with the levels of N\textsuperscript{IC-1} detected by immunohistochemistry and that other factors contribute to regulating NOTCH pathway activity in ACL. Lung cancers are significantly hypoxic compared with normal lung epithelia (13). A recent study has shown that HIF-1\textalpha potentiates CBF-1–mediated transcription through direct association with NOTCH-1/CBF-1 transcriptional complexes (14).

Thus, we investigated the NOTCH signaling pathway in two ACL lines (A549 and H1755) under hypoxia. We found that, in 1% oxygen, mRNA expression levels of the NOTCH downstream factors HES-1, HEY-1, and HEY-2 were increased compared with those measured in the same cells cultured in normoxia (Fig. 3A). Under hypoxia, HIF-1\textalpha increased as did the total amount of NOTCH-1; however, steady-state levels of N\textsuperscript{IC-1} showed only a modest increase (Fig. 3B), suggesting rapid activation followed by degradation. Other components of the NOTCH signaling pathway seemed unaffected by hypoxia (Fig. 3B). In agreement with protein levels, hypoxia induced NOTCH-1 mRNA expression (Fig. 3C), anticipated to further support NOTCH signaling because NOTCH-1 expression is under a positive feedback loop (15). To understand the biological function of NOTCH signaling in ACL lines grown in hypoxia, we inhibited the pathway using the γ-secretase inhibitor MRK-003 (11). When ACL lines were...
transduced with control lentivirus (Fig. 4 but had no effect under normoxia (Fig. 4). Tantantly, MRK-003 treatspecifically killed ACL cells under hypoxia (Fig. 4).

The expression levels of the mRNAs of the genes of interest were normalized to 18S rRNA. Relative expression is calculated as normalized level of each mRNA in hypoxia-treated cells compared with normoxic conditions (the latter are set as 1 arbitrary unit). Columns, relative expression (six independent measurements); bars, SD.

We show the results obtained in ACL cell lines A549 and H1755. Similar results were obtained in other two ACL cell lines (data not shown). B, hypoxia specifically up-regulates NOTCH-1 protein expression. Western blot analysis was done 48 h of either normoxic or hypoxic incubation. Note that the protein levels of total NOTCH-1, cleaved NOTCH-1, and HIF-1α are increased by hypoxia, whereas other components of the NOTCH signaling pathway seem unaffected by hypoxia. C, hypoxia up-regulates NOTCH-1 mRNA expression levels. The relative expression of mRNAs was determined as described in (A).

exposed to increasing concentration of MRK-003, we observed dose-dependent accumulation of N\(^{\text{ISC}-1}\) and corresponding loss of N\(^{\text{IC}-1}\) (Fig. 4), confirming that NOTCH-1 cleavage was inhibited. As a further control, we measured HES-1 mRNA expression levels in cells treated with MRK-003 under normoxia and hypoxia. We found that in hypoxia, MRK-003 treatment reduced the expression of HES-1 mRNA 682-fold (Fig. 4B), further confirming MRK-003-mediated NOTCH signaling inhibition. MRK-003 treatment caused a potent apoptotic response in ACL cells as early as 48 h after treatment (Fig. 4C, middle). This apoptotic response was reduced if N\(^{\text{IC}-1}\) was reexpressed in these cells through doxycycline-inducible lentivirus (Fig. 4C, top right), whereas doxycycline did not affect cells transduced with control lentivirus (Fig. 4C, bottom right). Importantly, MRK-003 treatment specifically killed ACL cells under hypoxia but had no effect under normoxia (Fig. 4D). Expression of N\(^{\text{IC}-1}\) in ACL cells exposed to MRK-003 in hypoxia did not rescue the totality of cells. This can be explained by the fact that \(\gamma\)-secretase inhibitors prevent the activation of all four NOTCH receptors (16) or to off-target effects. Thus, we used a genetic strategy by down-regulating NOTCH-1 under hypoxia using a RNA interference (RNAi) approach. A shN1 was cloned into the pLenti4/TO/V5-DEST and the ACL cell line A549 was transduced with this construct. When transcription of NOTCH-1 targeting small hairpin RNA was induced by doxycycline in this cell line under hypoxia, apoptosis resulted (Supplementary Fig. S5), further confirming that NOTCH-1 signaling is required for ACL cell survival under hypoxia.

Our data show that the biological outcome of NOTCH signaling in lung cancer depends on oxygen concentrations. When ACL cell lines were studied under standard tissue culture conditions, NOTCH-1 seemed to have tumor-suppressive activities, whereas under hypoxia (a condition that more closely reflects tumor physiology; ref. 13), NOTCH seems to be essential for cell survival. This may explain part of the controversy about the role of NOTCH signaling in cancer (6). Furthermore, our data suggested that the role of hypoxia should be carefully considered when reexamining earlier studies showing tumor suppressor activities for NOTCH (e.g., refs. 17–19).

The effects of NOTCH signaling are notoriously dose dependent (7). It is likely that mechanisms have evolved whereby cells with excessive NOTCH activity die, as is the case for other well-known oncogenes. An excess of N\(^{\text{IC}-1}\) is toxic for numerous cell types in vitro, and cells use multiple mechanisms to maintain optimal levels of NOTCH activity (7). In normal human keratinocytes, NOTCH-1 causes growth arrest at high levels and transformation at low levels (7). Under hypoxia, which potentiates the strength of NOTCH signaling, the low levels of N\(^{\text{IC}-1}\) protein may reflect rapid activation-degradation. Alternatively, ACL cells may reduce total NOTCH protein expression to prevent hyperactivation and maintain NOTCH signaling to levels compatible with life. Our data do not exclude a participation of NOTCH-3 in the pathogenesis of ACL. Indeed, cross-talk between NOTCH-1 and NOTCH-3 has been described in some systems (7). Nonetheless, NOTCH-1 signaling seems essential for survival of ACL cells under hypoxia. Our data suggest that targeting NOTCH signaling using \(\gamma\)-secretase inhibitors may be an attractive therapeutic strategy to treat highly lethal ACL and possibly other commonly hypoxic malignancies (20).

Acknowledgments


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Figure 4. Inhibition of NOTCH signaling results in cell death under hypoxia. A, treatment with the \( \gamma \)-secretase MRK-003 leads to accumulation of the (uncleaved) NOTCH-1 protein in a dose-dependent fashion. Cells were treated with MRK003 (at the specified concentrations) under either normoxia or hypoxia for 48 h. The transmembrane portion of NOTCH-1 was then detected using an antibody specific for the total (uncleaved) NOTCH-1 protein. B, treatment with MRK003 reduces HES-1 mRNA expression. A549-TR-N1 cells were treated with 40 \( \mu \text{mol/L} \) \( \gamma \)-secretase inhibitors (GSI) under hypoxia for 48 h. Real-time RT-PCR was done and quantitated as described in Fig. 3A. C, NOTCH inhibition through MRK-003 treatment causes ACL cells death under hypoxia. Cells were treated with 40 \( \mu \text{mol/L} \) \( \gamma \)-secretase inhibitors for 48 h, and then cell viability was determined by Annexin V/7-AAD staining. Note that MRK003 treatment leads to a \( \sim \) 3.6 increase in the amount of dead cells. Constitutively active NOTCH-1 reexpression in these cells leads to a \( \sim \) 2-fold reduction of dead cells despite MRK-003 treatment. Similar results were obtained using a RNAi-based strategy (see Supplementary Fig. S6). D, inhibition of NOTCH signaling results in cell death specifically under hypoxia. ACL cell lines A549 and H1755 were treated with 40 \( \mu \text{mol/L} \) MRK003 and then cultured either in normoxia or in hypoxia for 48 h. Cell viability was measured by trypan blue staining. Columns, viability (four independent experiments); bars, SD. Note that the \( \gamma \)-secretase inhibitor MRK-003 does not affect ACL cell viability in normoxia. Dox, doxycycline.

References


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